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THE TECHNIQUE OF TISSUE CULTURE "IN VITRO"

T. S. P. STRANGEWAYS

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BY THE SAME AUTHOR

TISSUE CULTURE IN
RELATION TO GROWTH
AND DIFFERENTIATION

C. H. Boisseau.

TECHNIQUE OF TISSUE CULTURE "IN VITRO"

By

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CAMBRIDGE

W. HEFFER & SONS LTD.

1924

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PRINTED IN GREAT BRITAIN

Preface

THE study of animal tissues by cultivation "in vitro" now constitutes an important branch of experimental biology, and it is owing mainly to the skilful and patient experimentation of Harrison, Burrows and Carrel that the technique has been evolved which has opened up this new and fruitful field of research.

The methods described in this work are those in daily use in the laboratory of the writer and have been found by experience to yield the most satisfactory results.

This manual is intended for the use of workers in various widely different sections of biological and medical science, and in consequence many of the directions may appear unnecessarily elementary to those familiar with aseptic and surgical technique. Such information has, however, been included in the hope that it may be of value to other investigators, such as zoologists, biophysicists and biochemists, who may not have the advantage of this knowledge but who nevertheless desire to undertake research for which tissue culture "in vitro" is required.

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Contents

		Page
	Introduction	ix
Section		
I.	Fittings for the culture room	1
II.	Fittings for the washing up and sterilising room	3
III.	Selection and preparation of apparatus for putting up cultures	6
IV.	To clean apparatus	9
V.	To sterilise apparatus	11
VI.	To clean and sterilise a Berkefeld filter	13
VII.	To filter fluids and extracts with a Berkefeld candle	13
VIII.	Handling sterilised instruments and apparatus	15
IX.	Cold storage of fluids and extracts	17
X.	To prepare saline solutions	18
XI.	Setting out the bench for the cultivation of tissues	24
XII.	On the selection of tissues and medium for cultivation	26
XIII.	To remove a chick embryo from its shell	27
XIV.	To remove an embryo from the uterus	29
XV.	To remove and cut up tissues for implantation	31
XVI.	To cultivate tissues in saline solution	33
XVII.	To prepare embryonic tissue extract	36
XVIII.	To put up cultures in embryo tissue extract and saline	39
XIX.	To sub-cultivate tissues growing in embryo extract and saline	39
XX.	To make a fine pointed gas flame	42
XXI.	To make a glass canula	42
XXII.	To prepare and sterilise simple saline solution	44
XXIII.	Setting out operating table and bench for the collection of plasma	44
XXIV.	Sterilisation and setting out of apparatus and instruments for collection of plasma	46
XXV.	To coat apparatus with paraffin wax	49
XXVI.	Operation for the removal of blood from carotid artery	51
XXVII.	To separate plasma from red blood corpuscles	56
XXVIII.	To transfer plasma to a capillary pipette	58
XXIX.	To obtain plasma from a vein of a bird	58

Section		Page
XXX.	To obtain blood from the heart	61
XXXI.	To obtain frog plasma	63
XXXII.	To obtain human plasma	66
XXXIII.	To cultivate tissues in plasma	68
XXXIV.	To cultivate tissues in plasma and embryo extract	69
XXXV.	To make sub-cultures of tissues growing in plasma or plasma and embryo extract	70
XXXVI.	The cultivation of frog's tissue in plasma	72
XXXVII.	Intra vitam staining of cultures	73
XXXVIII.	Intra vitam staining of mitochondria	74
XXXIX.	Fixation of cultures "in vitro" for staining	75
XL.	To make an aqueous solution of hæmatoxylin	76
XLI.	To make iron alum solution	76
XLII.	Scharlack R solution	76
XLIII.	Acetic Alcohol	76
XLIV.	Fixation in acetic alcohol and staining by iron hæmatoxylin	77
XLV.	To stain cultures for fat	78
XLVI.	To prepare Bouin's picric formol solution	78
XLVII.	Fixation of cultures by Bouin's solution and staining with iron hæmatoxylin	78

Introduction

THE technique for the cultivation of tissues "in vitro" is still in its infancy, and its importance in relation to experimental cytology is not yet fully appreciated. The results already obtained, however, are of extreme interest, and it is probable that in the future tissue culture will prove one of the most valuable methods of biological research.

Before a worker is able to employ this technique satisfactorily in his investigations he must first become thoroughly expert in the preparation and sterilisation of the apparatus and media required, and it has been the aim of the present writer to describe clearly and in detail various methods by which these preliminary processes may be carried out. The worker must make himself so familiar with the technique described in the sections dealing with sterilisation and aseptic precautions that the habit of handling sterilised instruments and medium in such a way as to prevent contamination becomes second nature. From the very first he should be careful to eliminate risk of infection in his operations and should carry out each step slowly and carefully. In this way he will in time acquire a habit of aseptic work which it will be difficult or impossible to lose.

The beginner may have considerable hesitation in deciding which medium and tissue he should employ, and the writer would therefore advise him to begin with saline solution and the embryonic heart muscle of the fowl. In this way he will acquire the necessary technique for the simple experiments, and at the same time he will be able to satisfy himself that the saline is made up correctly and with suitable salts. Of the three formulae for saline given in the text, the two first—Tyrode's and Drew's private formula—are, in the writer's opinion, the best. Drew's published formula has been included as it is widely quoted, and the worker may

therefore wish to try it. The importance of using absolutely pure salts is emphasised in the text, but as these are sometimes difficult to obtain it may be necessary to recrystallise in certain cases or to try salts from several sources before a satisfactory saline can be prepared.

The hydrogen ion concentration of the medium should lie between 7·4 and 7·8. Felton's colorimetric method for determining the hydrogen ion concentration of small amounts of fluid[1] may be conveniently used, but only an approximate measurement is obtained by simple methods such as this. The final proof of the suitability of the medium is the appearance of outwandering cells around the edge of the tissue fragment within 24 hours of implantation. Fragments of the ventricle of a 9-day chick embryo implanted in a properly prepared saline solution containing pure salts, show outwandering cells in 90–100 per cent. of the cultures.

The outwandering of individual cells into the surrounding medium by amœboid movement is the first change seen in the implant, and it is important to distinguish between mere outwandering and actual new growth. In many published papers dealing with tissue culture "in vitro" there seems to be some confusion between the two processes. The successive emigration of individual cells may give rise to a zone of tissue surrounding the original fragment, which will continue to increase in size for 48 hours or even longer, but true growth only takes place if there is multiplication of the cells by mitosis. If the medium is not wholly favourable outwandering may take place readily, whilst mitotic figures are rare or absent. Under really good conditions, on the other hand, outwandering is associated with extensive cell division. The most satisfactory medium for true growth is found to be embryonic extract in saline, with or without the addition of plasma. It should also be pointed out that it is possible to prepare medium which, while permitting both outwandering and mitosis to take place, is, nevertheless, unfavourable for *normal* growth. In such cases bi-nucleate and multinucleate cells are usually present. The extreme

[1] Felton, L. D., *Jour. Biol. Chem.*, 1921, xlvi, 299.

importance of this fact is obvious, since if the technique of tissue culture "in vitro" is to be applied to experimental research, the worker must be able to satisfy himself that the growth he obtains is normal, otherwise his results will be vitiated.

In connection with cultivation in plasma, it should be noted that the serum which separates out after clotting tends to inhibit growth in the course of a few hours. If, however, such cultures are changed every 24 or 48 hours, the toxic properties of the serum are overcome, whilst the cells also appear to acquire some degree of immunity to its action. In this way good, rapidly growing sub-cultures can be maintained indefinitely. It may sometimes be desirable to exclude the effect of serum after the clotting of the plasma, and this may be effected by allowing the clot to soak in saline for a short period, either before or after implantation of the fragment. As a rule, however, such precautions are unnecessary.

Another point in connection with tissue culture in plasma which should be borne in mind is that it is of some importance that the plasma should be obtained from a young animal, as it is found that growth is less favourable when an older individual is used for this purpose.[1]

The worker should be careful in his selection of the glassware to be employed in tissue culture. All glass used must be free from alkali, and should not be allowed to become overheated during sterilisation, otherwise successful growth will not be obtained.

In order to avoid confusion, directions for the use of media containing glucose, serum, egg albumen, agar, etc., have not been included in this text, as they have no advantage over the simpler methods described. The technique in which fibrinogen is used instead of plasma as a basis for implantation[2] and Carrel's methods of cultivation in glass flasks,[3] have also

[1] Carrel, A. and A. H. Ebling, *Jour. Exp. Med.*, 1921, xxxiv, 599.
[2] Carrel, A. and A. H. Ebling, *Jour. Exp. Med.*, 1923, xxxvii, 759.
[3] Carrel, A., *Jour. Exp. Med.*, 1923, xxxviii, 407.

been omitted, as any worker who has acquired the elementary technique and wishes to adopt these methods will be able to follow the directions given in the original papers.

The beginner will doubtless be tempted to modify the technique recommended in this work, especially if after a few trials he fails to obtain satisfactory results. The writer would, however, urge him to wait until he has succeeded in cultivating tissues by the methods described before he attempts any original improvements.

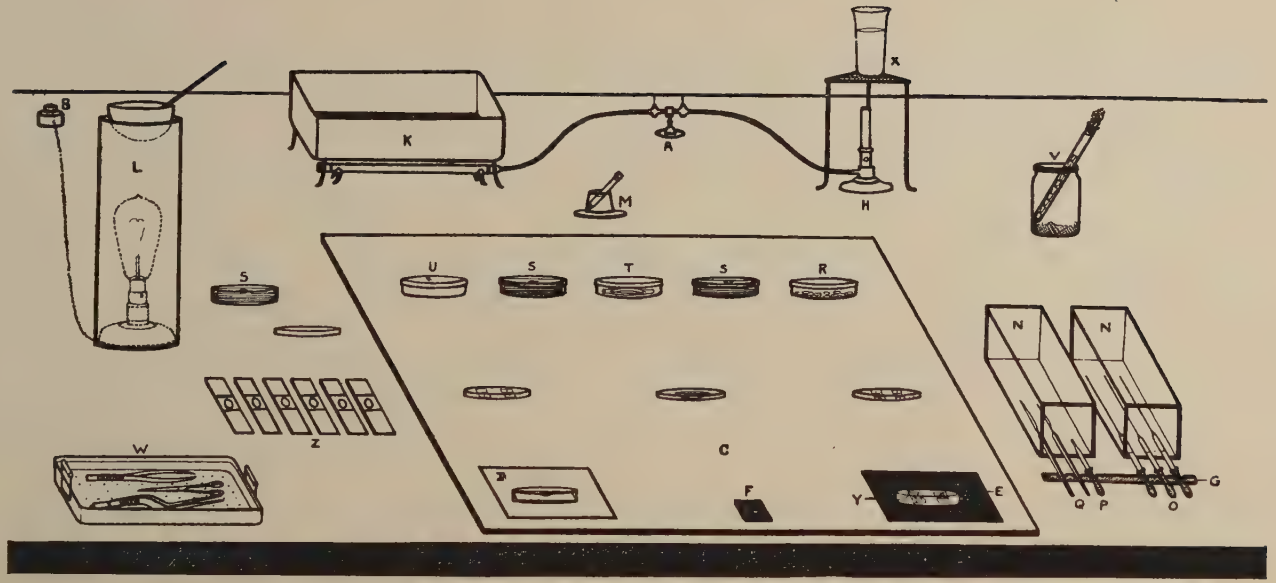


FIG. 1

The Technique of Tissue Culture "In Vitro"

SECTION I

Fittings for the Culture Room

For the successful cultivation of tissues "in vitro," the first essential is a small room with a good light and in which no other work is being carried on. The floor, benches and tables should be covered with linoleum. For the floor imitation oak linoleum No. 2 quality is suitable; for the benches and tables dark green smooth cork linoleum No. 2 quality.

The bench should be fitted with a 2-way gas tap (Fig. 1a) and an electric plug (Fig. 1b). On the right-hand side of the bench is placed a sheet of plate glass about 24 inches long and 18 inches wide (Fig. 1c), underneath which, on the left-hand side of the worker, a half sheet of white notepaper is laid (Fig. 1d), and on the right-hand a similar sheet of black paper (Fig. 1e). On the glass plate, about 2 inches from the edge, a small piece of black glass, about $1\frac{1}{2}$ inches square, is cemented with Canada balsam, and on the centre of this a small piece $\frac{1}{2}$ inch square is cemented (Fig. 1f). Pieces of glass cut from an exposed and developed photographic plate will answer admirably.

Some workers use a glass case about 24 inches long, 18 inches deep and 9 inches high. The top, sides and back of this case are made of plate glass, the front and bottom are open. This case is placed on a sheet of plate glass, and in it the apparatus is placed and the cultures are put up.

In laboratories where a separate room cannot be spared and in which other workers are present, such a piece of apparatus is essential, but if a room has been allotted for tissue culture work it is quite unnecessary.

On the right-hand side of the glass plate, about 4 inches from the edge of the bench, is laid a roll of plasticine, 10 inches long, and $\frac{1}{2}$ inch in diameter (Fig. 1g).

At the back of the glass plate a Bunsen burner with iron tripod and wire gauze is placed (Fig. 1h).

Further to the left there must be a small hot water steriliser for boiling the instruments (Fig. 1k).

On the left of the worker should be an electric lamp on a wooden base covered by a circular tin about 9 inches high and 3 inches in diameter from which the bottom has been removed. Into the upper opening of this a nickel or porcelain evaporating dish of suitable size is fitted (Fig. 1l); this contains the paraffin wax for sealing the cultures. If electric light is not available a Bunsen burner and tripod may be used, but unless the flame is carefully adjusted there is danger of overheating the wax in the evaporating dish, and thus causing it to catch fire.

Further along the bench should be a microscope with $\frac{2}{3}$ and $\frac{1}{6}$ objectives for the examination of cultures.

The room should also contain a Hearson incubator, a small covered electric or water centrifuge, and a chemical balance. There should be, in addition, a closed cupboard.

The culture room should be swept out and dusted each night before leaving. After dusting, a damp cloth should be passed over the bench and the glass plate covered with a clean, dry cloth. Any dust raised settles during the night, and the room may be safely used the next morning without further preparation. If the room is swept and dusted in the morning, contamination of the cultures is apt to occur. It should not be necessary to wash the floor of the culture room more than once a week unless dust accumulates in the room with exceptional rapidity.

For heating the room, radiators are preferable, and the ventilation must be so arranged that no draught passes over the working bench.

SECTION II

Fittings for the Washing Up and Sterilising Room

This room should be fitted with a bench with a 3-way gas tap, a large sink with draining board and a good supply of hot and cold water. On a shelf near the sink should be placed bottles of hydrochloric acid, sulphuric acid, bichromate of potash, test-tube cleaners, etc. On the bench a Bunsen burner with iron tripod and two enamel bowls; beneath it a galvanised iron waste bin. In this or in another room a large high speed centrifuge with buckets not less than $1\frac{1}{2}$ inches in diameter and $3\frac{1}{2}$ inches deep should be available.

The sterilisers required are:—

(1) Hot air oven for sterilising by dry heat (Fig. 2).

This is a small oven made with sheet iron, enclosed in a case of sheet iron on the underside of which a round hole is cut through which the flame of a Bunsen burner gives heat to the bottom of the oven. The heat passes up the sides and back and escapes by smaller holes at the top of the outer case.

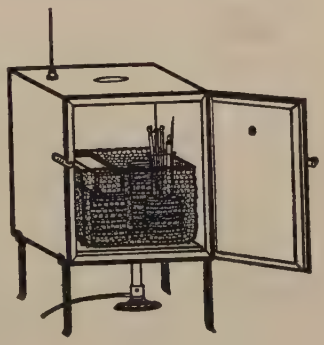


Fig 2

On the bottom of the oven should be placed a wire tray to prevent any article which is being sterilised coming into direct contact with the bottom of the oven.

These sterilisers may be purchased heated by gas or electricity, with or without an automatic temperature regulator, but a simple, movable oven heated by gas such as is supplied for kitchen use will answer admirably. In selecting this, however, care should be taken that the internal iron chamber contains no opening except the door and a small hole for the thermometer. The steriliser should

be about 12 inches square and 14 inches high, inside measurements. Arrangements must be made for a thermometer which registers 200° C. to pass into the interior of the chamber.

(2) Koch's steam steriliser (Fig. 3).

This consists of a tall cylindrical metal cylinder with a cover which is perforated with a small hole for a thermometer. Inside the cylinder a round perforated disc is fitted about 4 inches from the bottom. Water to a depth of about 3 inches is placed in the steriliser, and when in use the articles or solutions to be sterilised are placed on the perforated disc, the lid placed on with the thermometer in position, and the water brought quickly to boiling point by a large Bunsen burner. The advantage of this method of sterilisation is that no evaporation of any solution being sterilised occurs. 1½ hours steaming at a temperature of 100° C. will sterilise any fluid, but it is not necessary in many cases to give such a long steaming.

Fig. 3

(3) Autoclave for sterilisation by means of steam at high pressure (Fig. 4).

This consists of a cylindrical bucket of strong welded metal (Fig. 4a), inside which is brazed a cylindrical copper bucket perforated at the top so that steam may enter (Fig. 4b), These are enclosed in a sheet iron case (Fig. 4c) and with a cover which can be fastened down firmly by thumb screws (Fig. 4d). The cover is fitted with a safety valve (Fig. 4e), pressure gauge (Fig. 4f) and steam vent pipe (Fig. 4g). The welded bucket is fitted with glass water gauge (Fig. 4h). Sufficient water is placed in the welded bucket through the funnel at the top of the water gauge (Fig. 4k). The articles to be sterilised are put in a sterilising drum (Fig. 4l), which is placed in the steriliser. The top is then firmly screwed down. The valve of the steam vent pipe is opened and the water brought to the boil with the Bunsen burner

(Fig. 4m). As the steam comes off, the air is driven out of the steriliser, so that the chamber becomes filled with steam. The escape of clouds of steam from the steam vent pipe shows when the air has been driven out. The valve is then closed, and thus further escape of steam is prevented. The pressure in the chamber will now begin to rise, and the degree of pressure will be recorded on the pressure gauge. A good working pressure is about 30 lbs. to the square inch, which will give a temperature of 120° C. The safety valve must be carefully adjusted so as to blow off when the desired pressure is obtained; this should be tested each time the steriliser is used, thus avoiding the possibility of a serious explosion. It is important to be sure that all air has been driven out of the steriliser, as otherwise the reading of the pressure on the gauge will not be a true record of the temperature inside the steriliser. Before any attempt is made to remove the contents of the autoclave after sterilisation the gas must be turned off and the steam allowed to escape gradually by opening the steam vent pipe, and no attempt must be made to turn the thumb-screw of the cover until the pressure gauge registers zero. More elaborate autoclaves can be purchased, but a simple kind such as described will answer every purpose.

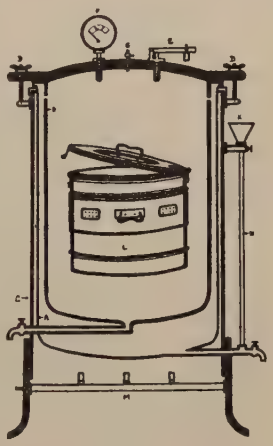


Fig 4

(4) Sterilisation by boiling.

For this a surgical hot water steriliser (Fig. 1k) is most convenient, although a small fish kettle will answer. The instruments and articles to be sterilised by this method are placed on a movable perforated tray (Fig. 1w), and well covered with distilled or rain water, to which a little bicarbonate soda is added in order to prevent rusting of the metal instruments. The articles should be boiled for 20 minutes.

SECTION III

Selection and Preparation of Apparatus for putting up Cultures

The apparatus required depends to some extent on the animal and medium selected, but the worker should have the following articles and apparatus at hand:—

(1) Knives. The exact shape and size of these depends largely on the fancy of the worker, but, personally, I prefer von Graefe's cataract knives (Fig. 5).

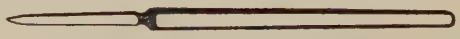


Fig. 5

The essential point is that the blade should be small and very sharp. Without a sharp knife good results cannot be obtained, and all knives must be sharpened after use. For sharpening, a good hone is necessary. An even, smooth and very light touch on the hone is required, and the stroke must be made against the edge of the knife from heel to point on either side of the blade alternately. After honing, the knife may be lightly stropped.

(2) Forceps. One pair of long curved forceps; Alder Smith's epilation forceps with curved ends are a suitable type (Fig. 6).

Fig. 6

Four pairs of dissecting forceps, two with blunt and two with fine points.

Fig. 7

One pair of Politzer's aural forceps (Fig. 7).

(3) Scissors. One pair of fine pointed scissors with the blades curved on the flat (Fig. 8) and a similar pair with straight blades.

Fig 8

One pair of scissors curved on the flat, with rounded ends (Fig. 9).

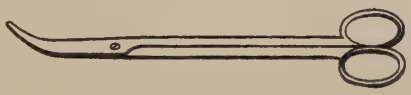


Fig. 9

(4) Petri dishes. $3\frac{1}{2}$ inches in diameter and 1 inch deep. One dozen (Fig. 10).

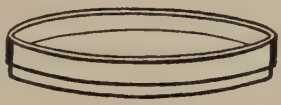


Fig 10

(5) Hollow ground slides. 3 inches by 1 inch, with deep well. Six dozen (Fig. 11). One of the ends should be

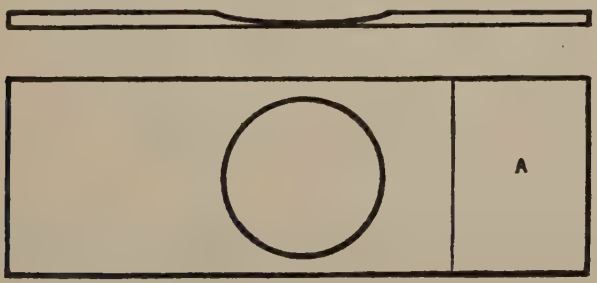


Fig. 11.

roughened for about 1 inch by rubbing with a piece of moist carborundum. On this ground surface details of the culture can be written in pencil (Fig. 11a).

(6) Coverslips. No. 1, $\frac{7}{8}$ of an inch square.

(7) Paraffin wax. Melting point 50° C.

(8) A porcelain or nickel evaporating dish, 2 inches in diameter.

(9) Small camel hair brush for sealing slides with melted paraffin wax.

(10) Capillary pipettes fitted rubber teat (Fig. 12). To make these, select glass tubing about 6 mm. in external

Fig 12

diameter. With a sharp glass knife cut into lengths of about 12 cm. Heat the centre in the blow pipe flame, rotating the glass tubing while heating it. When the centre is softened remove the tube from the flame and draw the ends slowly apart so as to increase the length to about 30 cm. and thus form a capillary tube. Hold the ends firmly apart until the capillary tube hardens, lay on the bench till cool, then divide in the centre with a sharp glass knife. In this way two capillary pipettes will be made, on the larger ends of which the rubber teats can be placed.

(11) Glass pipettes made with tubing of the same bore cut into lengths of 15 cm. Soften the centre as before in the flame, then remove from the flame, and after a second or two pull apart so as to make a capillary tube of about 2 mm. bore (Fig. 13).

Fig 13

When cool divide the capillary portion in the centre with the glass cutting knife, and thus two short large bore capillary pipettes will be made.

(12) One dozen 1 c.c. graduated glass pipettes. Select pipettes in which the graduation ceases some distance from the end in order to avoid the danger of drawing the liquids

too close to the lips. The upper end of the pipette should be plugged for about an inch with a small piece of cotton wool (Fig. 14).

(13) Measuring pipettes. 5–10–15–20 and 50 c.c.

(14) Graduated glass measures. 25–50–100–200–500 and 1000 c.c.

(15) A nest of beakers.

Fig. 14

(16) Two Jena flasks holding 2 litres for distilled water.

(17) Twelve Jena flasks 150 c.c. for saline solution.

(18) One dozen small centrifuge tubes fitted with corks to fit the small centrifuge.

(19) Two square glass museum jars, $8'' \times 3'' \times 2''$.

(20) Glass tubing, 6 mm. bore.

(21) One dozen watch glasses.

(22) Rubber teats to fit glass tubing.

(23) Cotton wool.

(24) A supply of new corks of various sizes.

SECTION IV

To Clean Apparatus

(1) Hollow ground slides.

(i) If previously used, remove any adherent paraffin wax in running hot water.

(ii) Cleanse thoroughly with some preparation of powdered soap such as Vim, by picking up the powder with the finger and thumb and rubbing the wet slide with it.

(iii) Wash in hot water and then in methylated spirit.

(iv) Dry and polish with a clean, dry glass cloth.

(2) To clean coverslips.

(i) If previously used remove the paraffin wax with the fingers, holding each coverslip in running hot water while doing so.

(ii) While wet clean thoroughly with Vim, taking the powder between the index finger and thumb.

(iii) Rinse well in hot water.

(iv) Place the coverslip in an acid bichromate solution made by adding 5 grams of potassium bichromate to 250 c.c. of a 5 per cent. solution of sulphuric acid in water.

(v) Drop each coverslip in the solution one at a time so that both surfaces come in contact with the solution. (If the solution is poured on to a mass of coverslips the glass surfaces adhere to one another, and the acid solution cannot reach them properly.)

(vi) Boil for one hour, adding fresh water when the solution has evaporated to about half its bulk.

(vii) Pour off the acid solution and wash each coverslip in running water, holding it in a pair of clean forceps. Be very careful to remove all traces of the bichromate solution.

(viii) Wash in two or three changes of distilled water.

(ix) Drop the coverslips into absolute alcohol in a wide-mouthed, closely-stoppered bottle, in which they should remain until required.

(x) When required the coverslips must be removed with clean forceps and wiped with a clean, dry glass cloth or between two small boards covered with dry muslin, which has been previously washed.

(xi) With a pair of forceps place the coverslips in a clean Petri dish; do not touch them with the fingers.

(3) Capillary pipettes should be made as described in Section III. from new glass tubing, and should not be used more than once.

(4) Graduated glass pipettes should be cleaned immediately after use by holding under a stream of hot water, then under a stream of distilled water, and finally washed out with pure alcohol and allowed to dry end upwards.

(5) The flasks for distilled water should on no account be used for anything else. They should occasionally be washed out with a little absolute alcohol and sterilised by dry heat,

and after cooling washed out with freshly distilled water before filling. They should be filled direct from the still, and have well fitting corks.

(6) Watch glasses, centrifuge tubes, test-tubes, Petri dishes and other glass-ware should be washed in hot water with Hudson's soap and rinsed in several changes of clean water, then with distilled water and dried with a clean, dry glass cloth. If it is necessary to use acid for cleaning be sure that all traces of acid are removed by rinsing out each tube or dish at least eight times under running water. If the centrifuge or test-tubes have been paraffined, the paraffin must be removed with very hot water and the tubes rubbed with a paper swab made by wrapping a piece of paper on the end of a piece of stiff wire or slip of wood.

SECTION V

To Sterilise Apparatus

(1) Glass apparatus may be sterilised by dry heat in a hot air steriliser (Section II. 1) at a temperature not above 130° C. for half-an-hour, and must be quite dry when placed in the steriliser. The various articles may be conveniently placed in a wire basket for sterilising. They are thus kept together and easily carried. The steriliser should be allowed to cool down before removing the apparatus. It is also important that the steriliser should be cool when the apparatus is placed in it, otherwise breakage may occur.

(2) To sterilise capillary pipettes. Place these inside a larger pipette, which is made by drawing out the end of a test-tube to a shape similar to that of the pipette, but with a larger bore.

The pipette is placed within this and the open end of the test-tube plugged with cotton wool (Fig. 15). This is sterilised in the hot air steriliser at 130° C. for half-an-hour. Several of these should be prepared.

(3) Cover glasses, hollow ground slides, watch glasses and small centrifuge tubes are sterilised in Petri capsules in the

hot air steriliser for half-an-hour at a temperature of 130° C.

(4) Small knives and cataract knives should be carefully dried after sharpening and placed in test-tubes with the blades upwards (Fig. 16).

The test-tubes are then plugged very tightly with cotton

Fig. 15

wool and sterilised in the hot air steriliser for half-an-hour at 130° C.

(5) Jena flasks, graduated pipettes, large centrifuge tubes, and glass jars should be wrapped in grease-proof paper and sterilised in the hot air steriliser for half-an-hour at 130° C.

Fig. 16

(6) Forceps, scissors and other metal instruments are sterilised by boiling for 20 minutes in a hot water steriliser (Section II. 4). A little bicarbonate of soda should be placed in the water to prevent rusting.

(7) Corks for plugging test-tubes and centrifuge tubes must be sterilised in the autoclave (Section II. 3) for 30 minutes at 120° C.

Fig. 17

Corks must be carefully selected to fit the tubes for which they are required, and then placed in test-tubes plugged with cotton wool. By this method several can be sterilised at once and kept for use when required (Fig. 17).

SECTION VI

To Clean and Sterilise a Berkefeld Filter

(1) Immediately after use disconnect the parts of the apparatus and place the candle in a mixture of equal parts of liquor sodæ chlorivatæ (B.P.) and a 15 per cent. solution of caustic soda.

(2) After standing for 24 hours in the above mixture remove the candle and fit up the apparatus, connecting the outlet tube of the vacuum flask to a vacuum pump.

(3) Fill up the container with a 15 per cent. solution of caustic soda and filter this through the candle into the flask. Repeat this.

(4) Disconnect the parts of the apparatus and wash thoroughly to remove all caustic soda from the surface.

(5) Connect up the apparatus again as above, fill up the container with distilled water, and filter this through the flask. Repeat this six times.

(6) Disconnect the apparatus and place the candle in a jar of distilled water where it is left until again required.

(7) Before using the filter again repeat operation No. 5.

(8) Disconnect apparatus, clean container and vacuum flask thoroughly, and finally rinse several times in distilled water.

(9) Connect up the parts of the apparatus loosely so that the rubber bung does not fit tightly into the vacuum flask, and the candle is not screwed up firmly in the container.

(10) Wrap the apparatus in stout paper and sterilise in the autoclave at 120° C. for 30 minutes. (Section II. 3.)

SECTION VII

To Filter Fluids and Extracts with a Berkefeld Candle

For filtering, a Berkefeld candle is required (Fig. 18a). These can be obtained in various sizes, and the size used depends somewhat on the quantity of fluid to be filtered.

If the quantity is small, the smallest size candles should be used.

The exact type of apparatus used will depend on the judgment of the worker. If several c.c. of fluid are to be filtered the glass mantle and container supplied with the candle (Fig. 18b and d) may be used. If only a few c.c. of fluid are available and it is desired to pass through the filter as much of this as possible, obtain a flat-bottomed glass test-tube which just fits over the side and top of the candle (Fig. 18c). In this way the fluid passes by the capillary attraction between the glass tube and candle, and practically

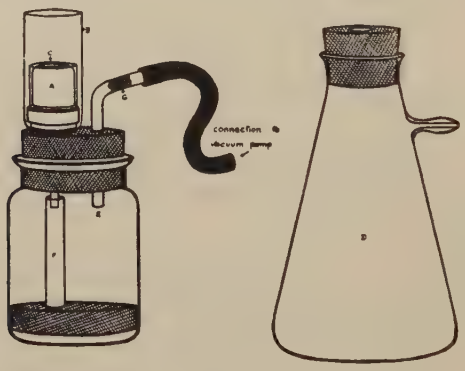


Fig. 18

all the fluid can thus be filtered. The object of using the glass cover is to enable a small quantity of liquid to cover the filter. If the quantity of fluid is considerable, the original flask may be used (Fig. 18d), but if a small quantity only is being filtered it will be found better to take a wide-mouthed bottle with a large 2-way rubber bung (Fig. 18e). Into this bottle is placed a small glass test-tube, the end of which it is convenient to fit into a perforated cork so that it stands immediately under one of the openings in the rubber bung (Fig. 18f). Through this opening the distal end of the metal mount is passed, deep enough for the mouth to pass into the top of the test-tube when the bung is placed in the bottle. Through

the other opening of the bung, a piece of glass tubing, about 4 inches long, bent at right angles, is inserted so that one end projects about half-an-inch into the bottle, the other end of course being outside. Into this end a plug of cotton wool may be inserted (Fig. 18g). The whole apparatus should be fitted loosely together (that is, the screws holding the filter in the mantle should be loose and the rubber bung should rest lightly on the mouth of the bottle) wrapped in paper and sterilised in the autoclave for half-an-hour. For use, fit the bung tightly into the wide-mouthed bottle in such a way that the opening of the metal end of the candle passes into the small test-tube. Pour the fluid to be filtered into the glass mantle. Exhaust the air from the bottle by connecting the bent glass tube by means of rubber pressure tubing to the exhaust nozzle of the pump which is supplied with the apparatus (Fig. 18). As the air is exhausted from the bottle the fluid in the glass mantle is sucked through the pores of the candle and passes into the small test-tube. When sufficient fluid has been filtered the pressure tubing is removed from the bent glass tube, the bung carefully taken out, the test-tube removed with sterile forceps and the opening closed with a sterile cork. The filtered extract is then stored in the ice chest or in a vacuum flask. (Section IX.)

SECTION VIII

Handling Sterilised Instruments and Apparatus

The worker must from the very beginning accustom himself to handling sterilised instruments and other apparatus in such a way as to prevent any parts which will come into contact with fluid or tissues used during cultivation from touching any unsterilised surface. It is probable that only experience will teach him how essential this is and make him realise the absolute necessity of re-sterilising any instrument or apparatus which has accidentally come into the slightest contact with an unsterilised

surface. If such an instrument or piece of apparatus is used, infection is almost inevitable; and much time and good material will be wasted if the worker, hoping to save either time or labour, uses again any article to which is attached the least suspicion of possible infection. The chief sources of danger will be found to be the following:—

(1) Capillary pipettes. When placing a teat on the bulb of a capillary pipette this should be taken by the finger and thumb of the left hand on the upper third of the bulb and the teat carefully placed in position without the capillary portion touching the hand, fingers, or sleeve, and the pipette should at once be placed in its proper position in the jar. When in use the capillary portion must never be allowed to touch anything but the fluid for which it is used. In removing fluids or extracts from test-tubes, watch glasses, etc., the outside and edges must never be touched. The pipette must be inserted into the various containers without touching the sides, and the same precaution must be taken on its withdrawal. If the pipette is to be used again it must be carefully replaced into its proper jar, with the utmost care, so that the sides and edges of the jar are not touched.

(2) Cataract knives must be so used that the blades, and at least one inch from the end of the handle, never comes into contact with any article which is not sterile, and it is even then generally safer to re-sterilise the blade by plunging it into the beaker of boiling water before returning it to its place in the glass jar. After such re-sterilisation always remember to allow the blade to become cool before using again, otherwise the hot blade may damage the culture.

(3) Sterilised forceps of various kinds should be laid on the edge of the tray in the hot water steriliser so that their points rest on the sterilised surface. These instruments may be re-sterilised by passing through the Bunsen flame, remembering to allow them to cool before use.

(4) Scissors of various kinds should be placed on the edge of the tray of the hot water steriliser in the same way as the forceps, and may be re-sterilised by dipping them into the beaker of boiling water again, remembering to allow them

to cool before use. But in the case of knives, forceps and scissors, such simple re-sterilisation is useless if they have accidentally touched an infected surface. When such an accident occurs they must be re-sterilised as described in Section V.

(5) Centrifuge tubes, test-tubes, watch glasses, Petri dishes, etc., must be kept sterile on their inner surfaces and edges. Sterile corks are always used, but the contents of a tube should on no account be allowed to come into contact with the cork. If by accident such contact should take place it is always better to reject the tube and its contents.

(6) Sub-cultures. Before sub-culture, tissues must always be examined under the microscope for possible infection. The cataract knives used for sub-culture should be carefully re-sterilised in the boiling water after transferring the culture to the fresh medium.

SECTION IX

Cold Storage of Fluids and Extracts

If a cold chamber or ice chest that can be relied on is available, fluids or extracts which are to be stored at zero should be placed in this. If such a cold storage chamber or ice chest is not available, small quantities can be conveniently kept on ice in vacuum flasks as follows:—

(1) By means of a sterile pipette, place the fluid or extract in a small sterile centrifuge tube or test-tube of about $1\frac{1}{2}$ cm. outside diameter and 8 cm. in length (Fig. 19a). Fit this tube with a sterile cork through which a thread has been drawn with a stout needle, and the ends then tied to form a handle (Fig. 19b).

(2) Place this in a container made of a test-tube about 2 cm. in diameter and 12 cm. long, fitted with a cork through the upper part of which a thread handle has also been drawn (Fig. 19c).

(3) The centrifuge test-tube and the glass container must be sterilised by dry heat. The corks already fitted with string handles are sterilised in the autoclave. (Section II. 3.)

Fig. 19

(4) Place the fluids or extracts to be kept in cold storage in the sterile centrifuge or test-tube. Cork with the sterile cork, and then place the tube in the sterile glass container, and cork this.

(5) Place some well-crushed ice into the vacuum flask and then with forceps carefully place the container by means of the thread handle on to the ice, and push it gently into the flask.

(6) Pack more crushed ice round the tube in the flask until it nearly reaches the cork.

(7) Close the flask with the cork, screw on the cup, and stand in the coolest part of the culture room.

(8) Each morning remove the glass container with forceps, pour off any water from the flask, replace the tube, and add fresh ice if required.

By this method the tubes and their contents can be kept cold for an indefinite period.

SECTION X

To Prepare Saline Solution

The selection and preparation of the saline solutions required for tissue culture is of considerable importance. A number of different formulæ have been given by different workers.

A

For warm-blooded animals Tyrode's solution, is recommended.

Tyrode's formula is as follows:—

Sodium Chloride	NaCl	0·8
Potassium Chloride	KCl	0·02
Calcium Chloride	$CaCl_2$	0·02
Magnesium Chloride	$MgCl_2$	0·01
Sodium Phosphate	NaH_2PO_4.	0·005
Sodium Bicarbonate	$NaHCO_3$	0·1
Distilled Water		100 c.c.

It is of the utmost importance that the salts should be pure. To make up the solution:—

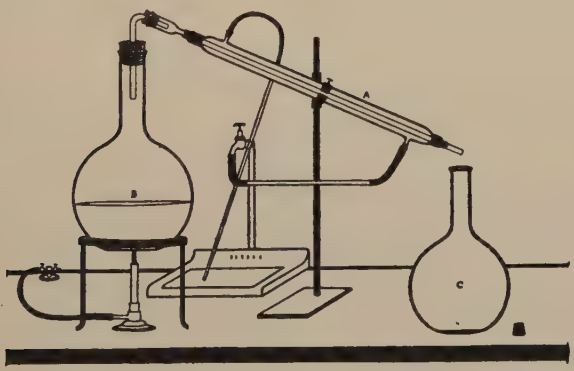
(1) Take a new 2-litre Jena flask, wash well in alcohol, and then in distilled water, dry carefully, and fit with a new clean cork.

(2) Sterilise the flask in the hot air steriliser at a temperature of 130° C. for 30 minutes (Section V. 1) and the cork in the autoclave in a bottle or large test-tube at a temperature of 120° C. for 30 minutes. (Section II. 3.)

(3) Distill in a glass retort, made by fitting a 30″ Liebig condenser (Fig. 20a) to a clean new 3-litre Jena flask (Fig. 20b) about 1500 c.c. of water.

(4) Re-distill about 1.200 c.c. of this directly into the 2-litre sterile Jena flask (Fig. 20c).

Fig 20



(5) When sufficient water has been distilled, cork the flask with the sterile cork until the water is required for use.

(6) Wash and dry thoroughly new graduated pipettes, holding 50 c.c., 5 c.c. and 1 c.c.

(7) Take a new 500 c.c. Jena flask and five other new Jena flasks holding 150 c.c each. Wash these thoroughly with distilled water, and dry carefully.

(8) Fit a new cork to each flask.

(9) Number the 500 c.c. flask No. 1, and the other five Nos. 2 to 6.

(10) Sterilise the pipettes and the six flasks in the hot air steriliser for 30 minutes at 130° C. Sterilise the corks in a suitable container in the autoclave.

(11) Remove the flasks from the steriliser and cork them with the sterile corks.

(12) Sterilise in the hot air steriliser six small squares of clean writing paper for 30 minutes at a temperature of 120° C.

(13) Weigh out the following salts in the order given on separate pieces of the sterile paper, and place each salt as it is weighed in the flask marked with the corresponding number:—

(i)	8 gram.	NaCl
(ii)	0·2 ,,	KCl
(iii)	0·1 ,,	$MgCl_2$
(iv)	0·05 ,,	$Na_2H_2PO_4$
(v)	1 ,,	$NaHCO_3$
(vi)	0·2 ,,	$CaCl_2$

(14) Remove the cork with the left hand from the flask No. 1, which contains 8 grm. Na Cl., and with a sterile 50 c.c. pipette place into it exactly 500 c.c. of the freshly distilled water. Replace the cork.

(15) In the same way put into each of the other flasks marked Nos. 2 to 6 exactly 100 c.c. of the freshly distilled water.

(16) Put the necessary quantity of water into the steam steriliser and place it over a Bunsen burner.

(17) Place the six flasks in the steamer, and with sterile forceps remove the corks and rest them horizontally over the mouth of their repective flasks.

(18) Put the cover on the steamer, insert the thermometer and light the gas.

(19) Bring the water in the steamer to the boil as quickly as possible, and steam for 10 minutes after the thermometer registers 100° C.

(20) Turn off the gas, take off the cover, and with a pair of sterile forceps replace each cork lightly into its flask.

(21) Remove the flasks from the steriliser and raise the corks once or twice as the solutions cool.

(22) When required for use, but not before, measure with a sterile pipette into a perfectly clean sterile Petri capsule 5 c.c. of the solution from flask No. 1 and 1 c.c. from each of the flasks numbered 2 to 6 inclusive. It is important that the $CaCl_2$ solution should be added last to the mixture.

If a larger quantity of the saline is required it must of course be made up in the same proportions.

It is not necessary to take new Jena flasks every time fresh solutions are required, but the flasks and pipettes must be kept for the solutions and used for nothing else.

B

The writer for many experiments uses a formula given to him privately by Dr. A. H. Drew, to whom I am indebted for permission to publish the formula, which is as follows:—

NaCl	0·9
KCl	0·042
$NaHCO_3$	0·020
$CaCl_2$	0·020
$CaH_4(PO_4)_2$	0·010
$MgHPO_4$	0·010
$MgSO_4$	0·010
H_2O	100 c.c.

To prepare this,

(1) Clean, sterilise and fit with sterile corks seven 150 c.c. new Jena flasks and number these 1 to 7.

(2) Weigh out the salts in the order given and place in a flask with a corresponding number.

(i) NaCl	9 grams.
(ii) KCl	0·42 ,,
(iii) $CaH_4(PO_4)_2$	0·1 ,,
(iv) Mg_2HPO_4	0·1 ,,
(v) $MgSO_4$	0·1 ,,
(vi) $NaHCO_3$	0·2 ,,
(vii) $CaCl_2$	0·2 ,,

(3) Add to each flask 143 c.c. of freshly re-distilled water.

(4) Place the flasks in the steam steriliser and steam for 10 minutes at 100° C. as described in Section X. A.

(5) Replace the corks in the flasks as described in Section X. A. Allow to cool.

(6) When the saline is required for use place an equal volume from each flask into a sterile Jena flask or Petrie dish.

C

Drew* has published another formula, which is as follows:—

Sodium Chloride	NaCl	0·900 grm.
Potassium Chloride	KCl	0·042 ,,
Magnesium Hydrogen Phosphate	$MgHPO_4$	0·010 ,,
Calcium Phosphate	$CaH_4(PO_4)_2 2H_2O$	0·010 ,,
Sodium Bicarbonate	$NaHCO_3$	0·020 ,,
Calcium Chloride	$CaCl_2$	0·020 ,,
Distilled Water		100 c.c.

(1) Distil 1200 c.c. of water into a clean 2-litre Jena flask (Section X., 1 to 4).

(2) Wash, dry, sterilise a new graduated pipette holding 50 c.c. and six new Jena flasks holding 150 c.c. fitted with corks. (Section X. A., 6–12).

(3) Weigh out on sterile paper the salts in the order given and place them into the flasks with corresponding numbers.

(i)	9 grm.	NaCl
(ii)	0·42 ,,	KCl
(iii)	0·1 ,,	$MgHPO_4$
(iv)	0·1 ,,	$CaH_4(PO_4)_2 2H_2O$
(v)	0·2 ,,	$NaHCO_3$
(vi)	0·2 ,,	$CaCl_2$

(4) Add 100 c.c. of freshly distilled water to each flask (Section X. A., 14, 15).

(5) Fit with new corks and sterilise a new Jena flask holding 100 c.c. and three others holding 25 c.c. Number these 7–8–9–10, and also clean and sterilise a new 10 c.c. pipette.

* *Brit. Jour. Exp. Path.*, 1922, Vol. III, p. 20.

(6) When the flasks are cool pipette into the larger one marked No. 7, 10 c.c. of each of these solutions NaCl, KCl and $MgHPO_4$ from the flasks numbered 1–2–3; add 40 c.c. of freshly distilled water.

(7) Put 10 c.c. of the solution No. 4 $CaH_4(PO_4)_2H_2O$ into the small flask No. 8, and into the flask No. 9 put 10 c.c. of the solution No. 5 $NaHCO_3$, and into flask No. 10 put 10 c.c. of solution No. 6 $CaCl_2$.

(8) Steam for 10 minutes in the steamer and cork (Section X. A., 16–21).

(9) Sterilise in the hot air steriliser a new clean Jena flask holding 150 c.c., fit with a sterile new cork, and when cool with a sterile pipette place the four solutions Nos. 7–8–9–10 into this, being careful to add No. 10 $CaCl_2$ solution last.

The saline solution is now ready for use and 100 c.c. of the solution is thus prepared every time it is required for use.

D.

For cold-blooded animals the following solutions are recommended. They should be prepared the same way as the saline solutions given above.

Ringer's Solution for cold-blooded animals

NaCl	0·7
KCL	0·25
$CaCl_2$	0·3
Distilled water	100 c.c.

Lewis' Solution for cold-blooded animals

NaCl	0·7
KCl	0·042
$CaCl_2$	0·025
$NaHCO_3$	0·02
Dextrose	0·25
Distilled Water	90 c.c.

SECTION XI

Setting out Bench for the Cultivation of Tissues

Apparatus required: 2 museum jars, 3 fine capillary pipettes, 2 large bore capillary pipettes, a graduated pipette, Petri capsules, 4 watch glasses, coverslips, hollow ground slides, 2 small centrifuge tubes, corks for centrifuge tubes, 2 cataract knives, 1 pair of dissecting forceps, 1 pair of fine forceps, 1 pair of fine curved scissors, 1 pair of curved scissors with rounded ends. All these must be carefully cleaned and sterilised. See Sections IV. and V. Paraffin wax in evaporating dish with small camel hair brush, saline solution.

Take into the culture room all apparatus and material required and shut and lock the door, otherwise stray visitors may cause infection of apparatus or cultures.

(1) Remove the paper from the two glass jars and place them on the bench about 1 inch behind the roll of plasticine (Fig. 1n).

(2) Take one of the glass tubes containing the fine capillary pipettes, remove the cotton wool plug, and with a pair of sterilised forceps take out the pipette. Put teat on pipette as described in Section VIII (1).

(3) Take this pipette by the teat between the thumb and index finger of the right hand and place it carefully in the glass jar furthest from the glass plate, allowing the larger end to rest upon the roll of plasticine (Fig. 1o). It is important that the capillary portion of the pipette should not be touched by the fingers or allowed to touch any article on the bench except the inner side of the sterile glass jar.

(4) In the same way prepare and place in the same jar the two other fine capillary pipettes.

(5) Fix a teat on the large bore pipette and place it in the glass jar nearest to the glass plate (Fig. 1p).

(6) Take the test-tube containing one of the cataract knives in the left hand, and with a pair of sterile forceps remove the cotton wool plug.

(7) Place one of the cataract knives in the glass jar nearest the glass plate with a pair of sterile dissecting forceps, so that the handle rests on the plasticine roll (Fig. 1q).

(8) Put out the second cataract knife in the same way.

(9) Place on the back of the glass plate the Petri dishes containing the coverslips (Fig. 1r), hollow-ground slides (Fig. 1s), watch glasses and centrifuge tubes (Fig. 1t) the special Petri dish reserved for saline solution (Fig. 1u), and the test-tube containing sterile corks to fit the centrifuge tubes (Fig. 1v).

(10) Place the forceps and scissors in the hot water steriliser, cover completely with distilled water, add a little bicarbonate of soda, and boil rapidly for 20 minutes (Fig. 1k).

(11) Just before the instruments are required for use remove the tray on which they lie from the steriliser and place it on the inverted cover of the steriliser in a convenient position (Fig. 1w).

(12) Nearly fill a glass beaker with distilled water and place it on the wire gauze of the tripod at the back of the glass plate and keep the water at gently boiling with a Bunsen flame (Fig. 1x).

(13) Place a little paraffin wax (melting point 50° C.) in the evaporating dish over the electric lamp, and melt this by switching on the light (Fig. 1l).

(14) Place an enamel pail just under the bench on the left of the glass plate to receive waste.

(15) Select a stool or chair of convenient height for comfortable working and place it in front of the bench opposite the glass plate.

(16) With the sterile graduated pipette make up the saline solution as described in Section X. and place this in the special Petri dish reserved for it.

SECTION XII

On the Selection of Tissues and Medium for Cultivation

The media in common use are

(1) Saline solution alone or with dextrose.
(2) Saline solution with embryo extract.
(3) Plasma alone.
(4) Plasma with embryo extract.

The selection of the animal from which the tissues for cultivation are obtained, and the form of medium used, depends upon the requirements of the worker or on the observations it is desired to make. The cultivation of tissues from each type of animal presents certain special difficulties, but those of some animals are much more difficult to deal with than others. The easiest tissues to work on are undoubtedly those obtained from embryos, and especially those from the embryo chick of seven to nine days' incubation, as the eggs are readily obtained throughout the year. It is, with experience, fairly easy to grow the tissues of adult animals, but it is necessary to sub-culture such tissues daily until the growth commences, as there is usually an interval of from three to seven days before any signs of growth can be recognised. When adult tissues have once begun to grow they continue to do so readily on sub-culture.

Each form of medium has its special advantages and disadvantages. Saline alone is simple to use, but is not favourable for growth after the first or second sub-culture.

Saline and embryo extract will, with practice, give a good growth and allow repeated sub-culture, and this medium is very suitable for the demonstration of cell structure by various staining methods; but the cultures must be handled with care, otherwise the medium will run over the surface of the coverslip.

Plasma gives a solid medium, and the cultures can be readily transplanted from one place to another. It is used

for animal tissues from which embryo extract cannot be obtained, such as frogs and man.

Plasma and embryo extract is a very good medium, but it takes longer to prepare than the other forms, and the cultures are more difficult to stain.

The choice of the saline solution used will depend on the experience of the worker and on the nature of the experiment, but the beginner is advised to use Tyrode's solution.

SECTION XIII

To Remove a Chick Embryo from its Shell

For incubation of eggs, Hearson egg incubators are very convenient, but it is possible to incubate eggs successfully in an ordinary biological incubator run at the blood temperature of the bird. Arrangements must be made, however, to keep the eggs sufficiently moist by placing a tray of water in the incubator, and care must be taken to turn the eggs each day. Write the date on which the egg is placed in the incubator on the shell.

If plasma medium is being used it is advisable, though not necessary, to use eggs of the same strain as that of the bird from which the plasma is obtained.

(1) Set out the bench as directed in Section XI.

(2) With sterile forceps place two sterile watch glasses on the left-hand side of the glass plate and cover each with a Petri dish. To the right of these place two hollow-ground slides covered in the same way.

(3) Take one of the fine capillary pipettes and half fill it with saline out of the Petri dish containing the sterile saline by gently raising the cover and inserting the capillary portion of the pipette into the opening between the two halves. The cover is thus kept over the saline and contamination from the air prevented. Be very careful also not to touch the outside of the Petri dish or any other article with the capillary portion of the pipette.

(4) When the pipette is half full withdraw it from the solution, replace the cover of the Petri dish, and, holding the pipette vertically with the capillary portion down, carefully release the pressure on the teat so that no fluid is sucked up into the teat itself. With a little practice the right proportion of air can be expelled before sucking up the saline, and thus air is prevented from bubbling through the fluid in the pipette.

(5) As soon as the teat has fully expanded return it to its place in the glass jar, resting the larger end, as before, on the plasticine roll.

(6) Remove an egg of about 8 or 9 days' incubation from the incubator.

(7) Take your seat on the stool close to the bench in front of the glass plate and bring the enamel pail to your left-hand side.

(8) Hold the egg, large end upwards, in the left hand, and with the back of the handle of an ordinary surgical scalpel crack the top of the egg with a series of gentle taps. This will cause a number of small cracks to form all over the upper surface.

(9) Place the scalpel on the bench, and with a pair of sterile forceps carefully remove the cracked fragments of shell from the upper end, being careful not to break the underlying membrane. With a little practice this is easily accomplished.

(10) With a pair of sterile forceps remove the white membrane covering the contents of the egg, taking great care not to allow any fragments of shell, dust or membrane to fall on the vascular membranes of the embryo which will be found lying under it. In this way a circular opening about 1 inch in diameter is made, and through it, if the egg is fertile, the embryo is seen lying within its vascular membranes.

(11) Note whether the embryo chick exhibits movements, as sometimes partial development followed by death of the embryo occurs. Such dead embryos are of course rejected and another egg opened.

(12) With a pair of sharp pointed curved scissors cut through the membrane round the embryo, being careful not to cut into the yolk. Tilt the egg so that the yolk projects slightly over the edge of the shell and keep the egg steady in this position until the embryo has been removed.

(13) With the scissors snip the yolk sac and allow some of the contents to flow into the pail.

(14) With a pair of sterile aural forceps take the embryo by the unbilical cord and lift it gently through the opening, taking the utmost care not to touch the sides of the shell. If any membranes are still adherent to the embryo, snip through these with the curved scissors.

(15) Place the embryo in one of the watch glasses lying under a Petri dish. A little practice is required to do this successfully, as young embryos, unless handled very gently, break up and drop back into the egg or on to the bench.

(16) Remove the large bore pipette and half fill it with saline solution, as described in paragraph 3.

(17) Remove the Petri dish covering the watch glass containing the embryo. With a pair of sterile forceps raise the embryo in the left hand above the watch glass, and with a stream of saline from the pipette wash off as much blood as possible.

(18) Transfer the embryo to the second watch glass and cover with the Petri dish.

SECTION XIV

To remove an Embryo from the Uterus

If the tissues of the mammalian embryo are required they should be obtained from a young embryo and removed from the uterus with strictly aseptic precautions.

(1) Select a female in which gestation is not too advanced.

(2) Set out the bench as described in Sections XI. and XIII.

(3) Two sharp scalpels and six Spencer Wells forceps will also be required, and may be sterilised with the other instruments in the hot water steriliser (Section II. 4).

(4) Place the animal under a flat bell jar of a suitable size.

(5) Pour some ether on a piece of cotton wool and place it under the bell jar. The animal will soon become unconscious and will fall on its side.

(6) Place a little cotton wool in a wide-mouthed bottle of such size that the head of the animal will pass easily into the bottle.

(7) Pour some ether on the cotton wool. Remove the animal from the bell jar and place its head inside the bottle.

(8) Place the animal on its back and nail it by its four paws on to a board.

(9) Moisten the abdominal surface with soft soap and water, and with a sharp razor shave the hair from the abdomen and thorax.

(10) Sponge the shaved surface with cotton wool moistened in alcohol.

(11) With a pair of dissecting forceps raise the skin of the abdomen and make an incision through the skin along the middle line from the sternum to the pelvis, being careful not to cut through the abdominal muscles.

(12) Clip a pair of Spencer Wells forceps (Fig. 26a) on the skin on one side of the incision and a second pair on the skin of the other side.

(13) With the aid of a scalpel reflect the skin on both sides and keep it well reflected by hooking the Spencer Wells forceps on to two nails driven into the board, one on either side of the animal.

(14) Take a fresh scalpel and a pair of sterilised dissecting forceps, raise the abdominal muscles with the forceps, and make a small incision along the middle line through the abdominal wall, being careful not to wound the intestine or uterus.

(15) Clip a pair of Spencer Wells forceps to the edges of the muscular wall on each side of the incision.

(16) An assistant should now hold these forceps so as to form a pouch in which the uterus will be seen.

(17) With a scalpel enlarge the incision so that the uterus can be readily lifted out. Care must be taken not to allow the uterus to protrude through the incision and come in contact with the exposed surface.

(18) With a pair of Spencer Wells forceps seize the uterine wall and carefully lift the uterus from the abdomen, being careful not to allow it to touch the outside surface of the abdominal wall.

(19) With a sharp sterile scalpel cut through the uterine wall at its junction with the vagina. Sever the ligaments with a scalpel, and lift the uterus into a sterile Petri dish or glass jar.

(20) Kill the animal by opening the thorax and perforating the heart with the point of the scalpel.

(21) With a pair of sterile scissors open the uterine wall and remove the embryos by cutting through the unbilical cord, placing each one as it is removed into sterile saline solution in a Petri dish.

The tissues of the embryo may now be used for cultivation or for making embryonic extract as described in Sections XVII., XVIII., XIX., XXXIII., XXXIV., and XXXV.

SECTION XV

To Remove and Cut up Tissues for Implantation

Set out the bench and remove an embryo from its shell or the uterus as described in Sections XIII. and XIV.

(1) With a pair of sterile forceps remove two of the hollow-ground slides from the Petri dish and place them, hollow side upwards, on the glass plate.

(2) Into the hollow of each slide place a few drops of saline solution from the capillary pipette in the jar. Replace the pipette in the jar and cover the hollow-ground slides with the Petri dish.

(3) Remove the Petri dish covering the embryo in the watch glass. Take a cataract knife from the second glass jar in the right hand and a pair of fine sterile forceps in the left and carefully dissect out the tissues it is desired to cultivate. (Any part of the embryo will grow, but for a beginner the heart is best.)

(4) To dissect out the heart lay open the thorax and abdomen with the blade of the knife, holding the embryo steady with the forceps. This incision will expose the contents of the thorax and abdomen, and the heart will be recognised by its pulsations and shape.

(5) Cut through the vessels at the base of the heart with the blade of the knife.

(6) Remove the cover of the Petri dish covering one of the hollow-ground slides. Transfer the heart with the point of the knife to the saline solution in the hollow and re-cover it with the Petri dish.

(7) Replace the Petri dish over the embryo, dip the knife in the boiling distilled water and replace it in the jar. Flame the forceps.

(8) Bring the hollow-ground slides containing the heart immediately before you and remove the cover.

(9) Take the two cataract knives from the jar, holding one in each hand.

(10) Fix the heart by the base with the point of one knife and divide it in halves by a clean longitudinal cut with the other. Wash one half in the saline to remove as much blood as possible, and with the point of the knife transfer it to the saline already placed in the second hollow-ground slide.

(11) With the blade of the knife in the right hand draw the heart out of the saline on the flat part of the slide, bringing over at the same time a little of the saline.

(12) Steady the heart with the point of one cataract knife and cut the ventricle into small fragments. The knife should be sharp enough to cut through the tissue by gentle pressure. Slicing cuts must be avoided. It is of the utmost importance that the knife should be very sharp, and that a clean cut should be made through the tissue, so that there shall be no

tearing of the tissue, dragging out, or crushing of the cells.

(13) The ventricle in this way is divided into a number of small fragments from ½ to 1 mm. square.

(14) Replace the cover of the Petri dish over the hollow slide.

Fragments of tissue may be cut and prepared in the same way for culture from any other part of the embryo, or from the sterile tissues of an adult animal.

SECTION XVI

To Cultivate Tissues in Saline Solution

Set out the bench as described in Section XI.

(1) With a sterile pair of fine dissecting forceps place four coverslips on the right-hand side of your glass plate over the piece of black paper lying under it (Fig. 1y)

(2) Take the unused pipette from the glass jar and draw up a little saline as before. Shake off any adhering drop and place gently in the centre of each glass coverslip a small quantity of the saline.

(3) With the blade of a sterile cataract knife transfer one fragment of the tissue to be grown to the centre of the drop, and with the point of the knife spread the drop of saline around the fragment. The saline must be spread out in a circle with a diameter of about ½ an inch, and must form a deep film, not a drop of fluid. It should be of such an amount that when it is spread, as directed, around the fragment the latter appears to project slightly above the surface of the fluid. In this way surface tension causes the fragment to lie closely applied on the surface of the glass. The size of the drop of saline is therefore of importance, and a little practice will enable the worker to judge the size required (Fig. 21). If too large a drop has been placed on the coverslip the excess of fluid may be removed with an empty sterile pipett.

Fig. 21

(4) In the same way implant a fragment of tissue on the other three coverslips.

The secret of the success of this operation, and the subsequent growth of the tissue, depends on the cleaning of the coverslips. If they are too clean or have been heated above 150° C. when sterilising the fluid will run all over the coverslip and it will be impossible to form a circular film of saline. If, on the other hand, they are at all greasy, the saline will not adhere properly to the coverslip, and instead of forming a film will run into a drop, and the fragment will tend to fall away from the surface of the coverslip into the fluid. With practice, and by carefully following out the directions given in Section IV. 2, it will be possible to obtain a surface on which the film will spread evenly.

(5) Replace the Petri dish over the hollow-ground slide, containing the remaining fragments of tissue and cover the four implants with a sterile Petri dish.

(6) Remove one of the hollow-ground slides from the Petri capsule with a pair of sterile forceps and hold it at one end between the thumb and finger of the left hand with the hollow upwards.

(7) With the camel hair brush draw two lines of the melted paraffin wax (Fig. 1z) transversely across the slide, one on each side of the hollow. Place this slide on the glass plate before you.

(8) Prepare three other slides in the same way, and replace the camel hair brush in the paraffin to keep it warm for future use. Do not lay it on one side, or when required again, time will be wasted in melting the adhering paraffin wax.

(9) Remove the Petri dish covering the four coverslips, and with a pair of the fine sterile forceps slide one of them carefully to the edge of the glass plate.

(10) Take up one of the coverslips by the edges between the thumb and finger of the right hand.

(11) By a circular movement of the right hand invert the coverslip and place it so that the film rests over the hollow on the glass slide and the two edges of the coverslip not held by the fingers rest on the lines of paraffin wax on either side of the hollow.

(12) Take the camel hair brush and touch one corner of the coverslip with melted paraffin wax. Wait a moment to allow this to cool, and thus fix the coverslip.

(13) Now paint a thin film of paraffin wax round the four edges of the coverslip so that the paraffin overlaps the edges about $\frac{1}{8}$ of an inch. This should be done rapidly so as to leave a thin film which will not overheat the coverslip and thus injure the tissue.

(14) Paint once more round the edges in a similar way. It is most important that the coverslips be hermetically sealed, otherwise evaporation will occur and the culture will not grow.

Fig 22

(15) Label the coverslip carefully with the date, tissue used, or any other details, by writing with a sharp lead pencil on the ground surface of the slide.

(16) In the same way put up and label the remaining three cultures. Place them on a small cardboard tray and put them in the incubator at the temperature of the animal whose tissues are being cultivated.

(17) After putting up the cultures required, clean all the apparatus and instruments and wash and dry them thoroughly as described in Section IV. Leave your bench tidy and ready for work when required again.

(18) Examine the cultures the next morning. If growth is present the culture may be stained or allowed to grow another 24 or 48 hours; but if new growth is seen, proceed as follows:—

(19) Set out the bench as before (Section XI.).

(20) Pass the blade of a sharp scalpel carefully under the paraffin wax immediately after removal from the incubator while the wax is warm. Take great care not to allow the film of fluid to spread over the coverslip, and also not to touch the film of saline with the blade of the knife or fingers.

(21) Lay the coverslip film side upwards on the small glass square on the glass plate (Fig. 1f).

(22) Take up a little saline solution in a sterile pipette and add one or two drops to the film on the coverslip. Replace the pipette in the jar.

(23) With a new sterile pipette gently draw off the saline from the coverslip. Reject the saline thus drawn off and replace the pipette in its jar.

(24) With the pipette first used place two drops of saline on the coverslip. Remove these again with the second pipette as before. By this method the fragment of tissue is washed with fresh saline solution.

(25) Now place one drop of saline solution on the culture.

(26) With the empty pipette remove any excess of saline so as to leave the tissue lying in a film of saline as described in paragraph 3 above.

(27) Prepare a hollow ground slide, invert the coverslip, seal with paraffin wax, and replace in the incubator as described in paragraphs 6 to 15 above.

SECTION XVII

To Prepare Embryo Tissue Extract

(1) Set out the bench as directed in Section XI., and follow directions from 1 to 16 inclusive.

(2) Remove an embryo of 8 to 9 days' incubation from the shell (Section XIII.).

(3) Dissect out and place aside any tissues or organs it is desired to cultivate, as described in Section XV.

(4) Again wash the embryo in saline, as described in Section XIII., 17, removing as much blood as possible.

(5) Take the pair of curved scissors with rounded ends and cut the embryo up into fine fragments. It is desirable to remove the eyes before doing this in order to avoid the presence of pigment from the choroid in the extract.

(6) With the large bore pipette add a little saline to the minced embryo.

(7) Take a small centrifuge tube, and with the large bore capillary pipette draw up the minced embryo and saline and place it in the centrifuge tube. Add additional saline if necessary so that the mixture fills two-thirds of the tube.

(8) Stir the mixture with the sterile glass rod, being very careful not to touch the mouth or sides of the tube, and not to allow the mixture to come within half-an-inch of the opening.

(9) Take the test-tube containing sterilised corks, take out the cotton wool plug with sterile forceps, and with the point of a sterile scalpel remove a cork from the test-tube. Replace the plug in the test-tube. Take the cork from the point of the scalpel with the sterile forceps and insert it into the centrifuge tube containing the embryo extract. The cork should project sufficiently far from the opening of the tube for it to be taken out with forceps.

(10) Take the centrifuge bucket and place the tube within it, being careful that the cork does not project too far, otherwise it will rest on the plate of the centrifuge as it gains speed.

(11) Take a second bucket from the centrifuge and place it in a spare centrifuge tube; place the two buckets one on either side of the scales, and with a capillary pipette, which should be kept for the purpose, add water to the empty centrifuge tube until the two tubes exactly balance.

(12) Remove any spare buckets from the centrifuge and place the two balanced buckets opposite each other in their respective slots.

(13) It is usually necessary, especially if much blood remains after the embryo has been minced, to wash the embryo two or three times. In order to do this centrifuge the minced embryo at a low speed of about 1000 revolutions per minute for about 30 seconds, and thus bring down the bulk of the embryo and leave the blood corpuscles suspended in the saline.

(14) Decant and reject the supernatant fluid with the large bore pipette and leave the minced embryo in the tube. If

blood is still seen with the minced embryo add fresh saline sufficient to fill two-thirds of the tube, and with the glass rod mix the minced embryo and saline thoroughly.

(15) Replace the cork and place the tube in the centrifuge bucket; balance the two buckets carefully as before. Again centrifuge the mixture at a low speed for half a minute, and decant the supernatant fluid as before.

(16) With a sterile pipette add a volume of saline solution equal to that of the minced embryo. Stir with the glass rod. and replace the cork in the tube.

(17) Freeze the minced embryo to a solid mass in a mixture of crushed ice and calcium chloride.

(18) Remove the tube from the ice and allow the contents to thaw in the warm incubator.

(19) Again freeze the minced embryo and again thaw completely in the incubator.

(20) Place the tube in the centrifuge bucket and balance very carefully against the second as before.

(21) Centrifuge at about 2000 revolutions per minute for several minutes until the supernatant saline is almost clear and slightly opalescent. This can only be determined by removing the tube from the bucket after stopping the centrifuge. If not sufficiently clear it must be returned and centrifuged until clear. It is very important to balance the two buckets carefully, and to be sure that the centrifuge is running evenly, otherwise it will be impossible to obtain a clear extract.

(22) When a satisfactory extract has been obtained place the centrifuge tube in a suitable stand on the glass plate, or if it is to be kept for future use store it on ice. The extract thus prepared may be kept for several days, but must always be stirred and centrifuged again before use.

SECTION XVIII

To put up Cultures in Embryo Tissue Extract and Saline

(1) To put up the cultures take the tissues or organs which have been set aside for cultivation and cut up into suitably sized fragments, proceeding as directed in Section XV.

(2) Take a second capillary pipette from the glass jar and carefully removing the cork from the tube containing the embryo extract, draw up a little of the supernatant fluid and add the desired quantity of extract to the drop of saline already placed on each coverslip. Carefully release the pressure on the teat and replace the pipette in the glass jar. It will be necessary for the beginner to experiment with different dilutions of extract by putting up cultures in saline, to which varying quantities of the extract have been added.

(3) With the blade of a sterile cataract knife transfer a fragment of the tissue to be grown to the centre of the mixture, and with the point of the cataract knife spread the fluid around the fragment in a circle of about $\frac{1}{2}$ inch diameter. With an unused sterile capillary pipette draw off the excess of fluid present so as to leave the fragment adherent to the surface of the coverslip as directed in Section XVI., paragraph 3.

(4) Now proceed as directed in Section XVI., paragraphs 4 to 16 inclusive.

SECTION XIX

To Sub-cultivate Tissues growing in Embryo Extract and Saline

(1) Put up the tissues and renew the tissue extract and saline daily until growth commences as described in Section XVI., paragraphs 19 to 27 inclusive.

(2) When the new cells are seen on the edges of the fragment the mixture of saline and extract should be renewed once or twice, and under favourable conditions the zone of

growth will increase in size considerably. After two or three days however the increase in size usually ceases, and it is then necessary to wash the fragment of tissue in saline and transfer it to a new coverslip as follows.

(3) Set out the bench as in Section XI.

(4) With a sterile pipette place a little saline solution into the hollow of a ground slide.

(5) Remove the culture from the incubator, and while warm remove the coverslip with the blade of a scalpel (Section XVI., No. 20).

(6) Lay the coverslip, film side upwards, on the small square of black glass.

(7) Holding the coverslip with the thumb and finger of the left hand, with a sharp cataract knife make a clean incision through the film of cells on the outer edge of the growth, leaving about two-thirds of the new cells attached to the fragment.

(8) With the left hand turn the coverslip for a quarter of a circle, and with the cataract knife make another incision at right angles to the first through the film of new cells, again about two-thirds from the fragment.

(9) In a similar way make two more cuts at right angles through the film of cells.

(10) In this way the culture has been cut into a square fragment in the centre of which lies the original implant surrounded by two-thirds of the area formed by new cells.

(11) Replace the scalpel in the jar.

(12) With a sterile pipette allow two drops of saline solution to fall gently on the culture.

(13) Replace the pipette in the jar.

(14) With the blade of a sterile cataract knife very gently raise the fragment and as many of the new cells as possible from the coverslip and allow it to float free in the saline solution.

(15) Place the broad part of the blade of the cataract knife under the fragment and gently lift it from the drop of saline, keeping it spread out on the blade, and also avoid as far as possible detaching any cells.

(16) Very gently immerse the culture in the saline solution already put out in the hollow glass slide, and with gentle movements of the knife bathe the fragment in the saline.

(17) Put out a clean sterile coverslip over the piece of black paper under the glass plate, and on this place two drops of saline solution.

(18) With the blade of the cataract knife gently transfer the tissue to the drop of saline on the coverslip, being careful that it is spread out.

(19) With an empty sterile pipette draw off the saline on the coverslip so as to allow the fragment of tissue to float out on the centre of the surface of the coverslip. With care, the fragment will float out so that the original implant will lie upon the glass surrounded by the new cells.

(20) With an empty pipette draw off all excess of saline solution.

(21) With a fresh pipette make up a mixture of the saline solution and extract of the desired proportions on a sterile hollow-ground slide, and with the pipette gently place two drops of the mixture upon the fragment, avoiding, if possible, any displacement.

(22) With the cataract knife spread the mixture around the implant in a circle of about $\frac{1}{2}$ inch diameter.

(23) With an empty pipette remove the excess of the mixture so that a film of fluid remains as described in Section XVI., 3, 4.

(24) Prepare a hollow-ground slide, invert the coverslip, seal with wax, label and incubate as described in Section XVI., 6 to 18.

(25) When the technique has been acquired it will be possible to keep the implant growing for many sub-cultures, and with some tissues the number of possible sub-cultures appears to be indefinite.

SECTION XX

To make a Fine-pointed Gas Flame

(1) Take a piece of glass tubing about 8 inches long and draw it out at one end so as to make a short but fine capillary.

(2) With a sharp glass-cutting knife divide the capillary at its finest portion.

(3) Heat the stem about 2 inches from the larger opening in a fish-tail jet, and bend it carefully so as to form a right angle.

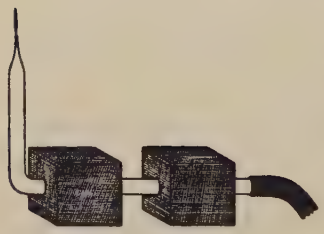


Fig. 23

(4) Fix this pipette end upwards in a holder, which can easily be made of a piece of lead or cork (Fig. 23).

(5) Place a piece of rubber tubing on the larger end and attach to the gas tap.

(6) Stand the burner thus made upright on the bench, turn on the gas and light.

(7) This should give a small flame about $\frac{3}{8}$ of an inch in height, the size being regulated by the bore of the pipette and the gas pressure.

SECTION XXI

To make a Glass Canula

(1) Take a piece of glass tubing about 6 inches long and about 3 mm. in diameter with a bore of about $2\frac{1}{2}$ mm.

(2) With a fish-tailed gas jet draw out a fairly fine capillary, about 1 mm. in diameter, outside measurement.

(3) Gently heat in the small, fine pointed gas flame the capillary portion towards the shoulder on the left-hand side where it is about $1\frac{1}{2}$ mm. in diameter, and by gentle manipulation make a flange.

(4) In the same way form a second flange in the capillary towards the right shoulder.

(5) Now gently heat the capillary portion of the tube about 2½ to 3 mm. from the flange formed on the left-hand shoulder, and with a steady movement draw this out as a fine capillary thread, keeping the whole tube firm and straight, and, if possible, avoid separating the two portions.

(6) Now heat the capillary portion about 2½ mm. from the flange on the right and again draw a straight capillary thread.

(7) With a sharp writing diamond cut off the capillary thread on the left about 2 mm. from the flange, and in this way a glass tube with a fine nipple-shaped nozzle will be formed. The lumen of the nipple of the nozzle should be not less than ½ mm. (Fig. 24).

FIG. 24

(8) With a writing diamond cut off the thread capillary from the other tube and thus form a second canula, which also has a nipple-shaped nozzle the lumen of which should be about ½ mm.

(9) Heat the wide end of the canula about 1 inch from the end in the fish-tailed jet, rotating carefully, and allow it to fall by its own weight so as to form an obtruse angle with the main body of the canula (Fig. 24).

(10) Bend the wide end of the other canula in the same way.

It is necessary to regulate the size of the nipple of the nozzle to correspond with the size of the vessel for which it is required, and several should be made so that a selection of various sizes is available, and thus at the operation it will be possible to choose one suitable to the lumen of the exposed artery.

SECTION XXII

To Prepare and Sterilise Simple Saline Solution

Add 9 grams of sodium chloride to 1000 c.c. of distilled water in a 2-litre glass flask, plug with cotton wool, and place in the autoclave and sterilise at 120° C. for half-an-hour (Section II., 3).

SECTION XXIII

Setting out the Operating Table and Bench for the Collection of Plasma

The blood may be conveniently obtained from the carotid artery, and by this method a large supply of plasma sufficient for several days may be stored.

For the beginner a fowl is the most suitable. It should be from 8 to 12 months old, although older birds may be used.

Whatever animal is used it is desirable that no food should be given for 24 hours before the removal of the blood, but plenty of fresh water must be allowed.

Selection and preparation of apparatus required for the operation.

Fig 25

(1) Place a small, firm table in a good light, and on this lay an operating board, which may be made from a piece of 1 inch plank 9 inches wide, and about 2½ feet long (Fig. 25). Into each of the longer sides, about 4 inches from each end, drive two nails at an acute angle (Fig. 25a). At one end of the board place a piece of wood 6 inches long and 2 inches square (Fig. 25b).

(2) Prepare two sandbags by filling two linen bags, 9 inches long, and holding about a quart, with dry sand, and close

the ends tightly. Lay these alongside the operating board. Four 2-feet lengths of ordinary 2-inch roller bandage for fixing the wings and legs of the bird are also required.

(3) Place on the table a bottle of anæsthetic ether, and a 2 lb. glass jar, at the bottom of which is placed some cotton wool. By the side of the table place an enamelled pail for receiving feathers, etc.

(4) Put out six pairs of Spencer Wells forceps (Fig. 26a), two pairs of Dieffenbach's artery forceps (Fig. 26b), one blunt probe (Fig. 26c), six towel clips (Fig. 26d), one pair of

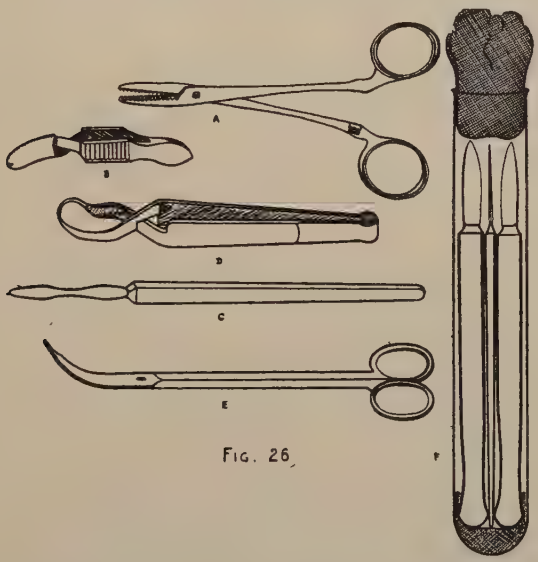


FIG. 26.

iredectomy scissors (Fig. 26e); these may be either blunt or fine pointed, but must be very sharp and the blades set close, and four small sharp scalpels in test-tubes (Fig. 26f).

(5) Make six glass canula (Section XXI.) and immerse them in pure olive oil in a large test-tube, which should be corked (Fig. 27).

(6) Select six perfectly clean, dry centrifuge tubes and fit these with corks. The centrifuge tubes must be considerably smaller than the bucket of the centrifuge in order to allow ice to be packed around them.

(7) Prepare four capillary pipettes in glass containers (Fig. 15), four rubber teats, two glass dishes, glass tongue jars will serve, two Thermos flasks, with test-tubes in glass containers for cold storage (Section IX.), a small pie dish, and an evaporating dish.

Fig. 27

(8) In a linen bag place twelve small gauze swabs. Take the corks which have been fitted to the centrifuge tubes and the test-tubes for the Thermos flasks and tie them in a bundle in a square of gauze.

(9) Fold separately four smooth clean towels.

(10) Place a glass flask containing simple sterilised saline (Section XXII.) on a tripod over a Bunsen burner.

SECTION XXIV

Sterilisation and setting out of Apparatus and Instruments for Collection of Plasma

Collect the apparatus and instruments as described in Section XXIII.

(1) Place in the drum of the autoclave the six glass canulæ in the olive oil in a test-tube, the linen bag with twelve gauze swabs, the bundle of corks, and the four towels. Sterilise all these at 125° C. for half-an-hour (Section II., 3).

(2) Place the four small scalpels in a test-tube and plug tightly with cotton wool.

(3) Place the four small scalpels in test-tubes, the capillary pipettes in containers, the centrifuge tubes and the test-tubes in glass containers for cold storage in a wire basket and sterilise in the hot air steriliser for half-an-hour at 130° C. (Section II., 1).

(4) Sterilise by boiling for half-an-hour the various forceps, probe, and iredectomy scissors (Section II., 4).

(5) Remove the drum from the autoclave, open it, and with a pair of sterile forceps remove one of the folded towels, and with the aid of another pair of sterile forceps open and lay it folded in half on the bench.

(6) Remove from the wire basket a capillary pipette in its glass container. Raise the upper fold of the towel, and place the pipette under the folds by removing the cotton wool from the container and tilting it gently, and thus allow the pipette to slide out on to the sterile towel. Place the other six capillary pipettes alongside this in the same way.

(7) With sterile forceps place the centrifuge tubes alongside the pipettes.

(8) With sterile forceps take the two test-tubes to be used for cold storage from their containers, and lay them under the folds of the towel, placing the containers alongside them.

(9) Remove the bundle of corks from the drum of the autoclave with sterile forceps and lay it under the fold of the towel.

(10) Paraffin the pipettes, the centrifuge tubes and the test-tubes into which the plasma is to be decanted for cold storage (Section XXV.).

(11) With the aid of sterile forceps fit the sterile corks into the paraffined centrifuge tubes, and into the test-tubes for the storage of plasma, replacing each under the fold of the towel as before.

(12) Take some powdered ice and fill the two glass jars. The ice is readily crushed by placing it between the folds of a thick, clean towel and pounding with a wooden mallet.

(13) Place in the ice in one of these jars, the centrifuge tubes, and in the other jar place in ice the test-tubes for cold storage of plasma, being very careful that the ice does not come into contact with the cork or mouth of the tubes (Fig. 28).

(14) Fit four rubber teats on the paraffined capillary pipettes (Section VIII., 1) and replace them in their respective containers with the rubber teats projecting from the opening and lay them on powdered ice in a small pie-dish, being careful not to allow the ice to touch the teat or opening of the container.

(15) With two pairs of sterile forceps take another sterile towel from the drum of the autoclave and lay it folded in half on the bench beside the operating board.

(16) With a pair of sterile forceps remove the bag of sterile swabs from the drum of the autoclave, open it and lay it with the swabs between the folds of the towel.

(17) Place the sterile evaporating dish for containing warm saline between the folds of the towel with sterile forceps.

(18) Remove the large test-tube containing the canulæ in oil from the drum of the autoclave, and with a pair of sterile forceps lay them one by one between the folds of the sterile towel.

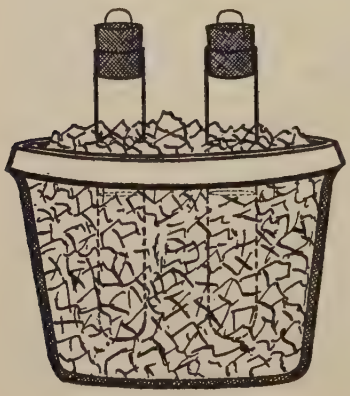


Fig. 28

(19) See that the centrifuge runs smoothly and place the buckets and balance ready for use.

(20) Light the Bunsen burner under the flask of sterile saline and heat this to a temperature of about 60° C.

(21) If a second room is available the small table with the operating board, sand bags and length of roller bandage, bottle of ether and the jam jar should be carried into this and the fowl then prepared for the operation outside the operating room in order to prevent dust and feathers flying when exposing the vessel for operation.

Everything is now ready for the operation necessary for obtaining the blood.

SECTION XXV

To Coat Apparatus with Paraffin Wax

(1) Carefully clean (Section IV.), dry, and sterilise in a wire basket in the hot air steriliser (Section V., 1) the glass apparatus it is desired to paraffin.

(2) Obtain a small enamelled milk can with removable

Fig. 29

lid holding about 1 pint (Fig. 29), and fill this three parts full with paraffin wax, melting point 56° C.

(3) Place the can and wax in the autoclave, with the lid resting on the mouth of the can, but not tightly closed, and sterilise for half-an-hour at 125° C. (Section II., 3).

(4) Fold a clean towel and sterilise it in the drum of the autoclave at 125° C. for half-an-hour. If any corks are required care must be taken that they are clean and fit the apparatus, wrap them in a piece of gauze, and place in the drum of the autoclave with the towel.

(5) Sterilise by boiling (Section II., 4) a pair of sponge-holding forceps (Fig. 30) and two pairs of dissecting forceps.

(6) Take the drum containing the towel and corks into the culture room, and with a pair of sterilised dissecting forceps remove the towel from the drum.

(7) With a second pair of sterilised forceps take the towel

by the edges and open it in such a manner that the folded halves shall not open, and lay it thus folded on the bench.

(8) Place the wire basket containing the capillary pipettes and other apparatus on the bench beside the towel.

(9) With a pair of sterilised forceps raise the upper fold of the towel and place the pipettes and any other apparatus between the folds. If any apparatus has been sterilised in paper covers the paper must be removed with sterilised forceps before placing the articles between the folds of the towel.

(10) If corks are required remove the bundle from the drum of the autoclave and place it between the folds of the towel. Keep all the apparatus covered until everything is ready for paraffining.

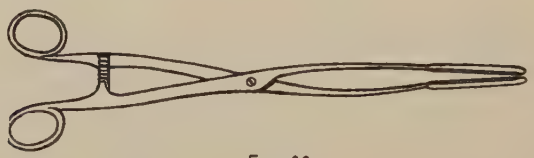


Fig 30

(11) When the paraffin wax has been in the autoclave for 30 minutes allow the steam to escape slowly through the vent. If the pressure falls too rapidly the wax will be blown out of the can.

(12) When the pressure has fallen, open the autoclave and take the can containing the melted paraffin to the culture room and place it on the bench beside the folded towel.

(13) Remove the lid of the can containing the hot paraffin wax.

(14) With a pair of sterilised forceps raise the upper fold of the towel with the left hand. Take the sponge-holding forceps in the right hand.

(15) With the sponge-holding forceps seize the bulb of one of the capillary pipettes in such a manner that it is held at an obtruse angle with the length of the forceps and dip it, capillary end downwards, into the melted paraffin wax so that it is completely immersed and filled with wax.

(16) Hold it for a second in the hot wax to warm the glass, then lift it out and invert the pipette quckly but carefully above the mouth of the can and allow the paraffin to run out rapidly through the bulb into the can.

(17) Hold it for half-a-minute in the air to allow the wax to set and then return the pipette to its place under the folds of the sterilised towel.

(18) If the lumen of the pipette is not patent or the coating of wax is not even, dip the whole pipette into the paraffin wax again and hold it there for a few seconds. The previous coating of wax will melt. Remove the pipette and invert it as before. By this method the bulb and capillary portion of the pipette will receive a thin, even coating of paraffin wax both internally and externally, and yet leave the lumen of the pipette quite patent.

(19) Any form of glass apparatus may be thus paraffined and placed ready for use under the folded sterile towel.

SECTION XXVI

Operation for Removing Blood from the Carotid Artery

(1) Catch the fowl gently, not frightening it more than can be avoided, carry it into the room in which the operating board has been placed, and lay it gently on its back on the board.

(2) Pour about half an ounce of ether upon the cotton wool in the glass jar. Put the head of the bird into the jar. (It is desirable for an assistant to hold the legs and wings, although if the bird is carefully handled and the anæsthetic given skilfully there is little or no struggling.)

(3) The jar should be removed from the head two or three times for a second or two in order to allow a fresh supply of air. The bird soon begins to breathe deeply. The animal's comb should remain a bright red colour the whole time it is under the anæsthetic. If it becomes dark the anæsthetic is being unskilfully administered. Anæsthesia

is complete when the bird begins to snore and shows no response to a light touch on the cornea.

(4) Take a length of roller bandage, and by means of a clove hitch round the junction of the wing with the body, fix one wing firmly to one of the nails on the upper part of the board, and in a similar way fix the other wing and both legs to the other nails. The bird is thus fixed in a spread-eagle manner on the operating board.

(5) Place on the outstretched wings the two sandbags.

(6) Place the neck of the bird upon the square piece of wood so as to raise it from the operating board.

(7) Pluck all the feathers from the upper part of the breast and the front of the neck so as to expose the whole of the front and sides of the neck. As the feathers are plucked they should be placed in the pail by the side to avoid scattering.

(8) The neck is now sponged with a little alcohol on a piece of gauze; then a piece of lint or gauze about 3 inches square is moistened with alcohol and laid on the exposed surface.

(9) The operating table with the fowl and anæsthetic bottle is then carried into the culture room and placed beside the towel containing the instruments, etc., so that a good light falls on the exposed neck of the bird.

(10) The assistant who has charge of the anæsthetic must keep the bird under complete anæsthesia during the whole of the operation. It is very important not to allow any movement of the bird while the blood is being taken.

(11) The operator now washes his hands thoroughly. He may wear rubber operating gloves, but these are unnecessary, and interfere with his touch. If the hands are well scrubbed and washed with soap and afterwards sponged with alcohol, there is no fear of contamination if the operation is properly carried out.

(12) Take a sterilised towel, fold it longitudinally, and lay it on one side of the fowl, and lay another sterilised towel in the same way on the other side. These should completely cover the bird with the exception of the exposed surface of the neck and head.

(13) With the towel clip fasten the adjoining edges of the towel to the lowest part of the exposed surface on the breast of the bird and be sure that no feathers are projecting above the edges. Remove the piece of gauze from the neck.

(14) Turn back the upper fold of the sterilised towel covering the instruments, and leave these exposed ready for use.

(15) Place the glass jar containing the paraffined centrifuge tubes in a convenient position.

(16) Take a sterilised scalpel and make a clean incision about 3 inches long down the front of the neck of the bird. This incision should be made with a clean sweep of the knife, which is then placed aside and not used again.

(17) Take a pair of Spencer-Wells' forceps and clip the edges of one of the sterilised towels to the middle of the incised skin. In the same way clip the skin on the other side of the incision to the edge of the other towel.

(18) In the same way clip the skin at the top and bottom of the incision with forceps to the edges of both of the towels, and by gently pulling on the forceps expose fully the underlying tissues of the neck.

(19) With a fresh sterilised knife make a careful incision down the middle line of the neck and through the fascia covering the carotid arteries, which will be found running together over the centre of the cervical vertebræ. The larynx and œsophagus are easily pressed to one side.

(20) With the blunt probe and occasional touches of the knife carefully dissect the arteries from their sheath and surrounding fasia, both in front and behind, so that they can easily be lifted up and the index finger placed under them.

(21) With the blunt probe carefully separate the two arteries from one another for a distance of about 1½ inches; place the index finger of the left hand under one of these and clip the distal portion with a pair of bulldog artery forceps, keeping the index finger under the artery. Pour a little warm saline into the sterile glass dish and moisten the vessels with saline.

(22) With a sterile swab sponge away any blood from the exposed tissues.

(23) Select two or three canulæ, the nozzles of which are most suitable for the size of the lumen of the exposed artery, and shake out any oil that remains in them. (The vessels in different birds vary somewhat in calibre, hence the necessity of having nozzles of different sizes.) Place the selected canulæ on the sterilised towel within easy reach.

(24) Compress the vessel between the thumb and index finger, and by slightly tilting the hand use the side of the tip of the index finger as a support for the vessel.

(25) Take the pair of iredectomy scissors and make a small nick so as to cut through about one half of the circumference of the vessel. Keeping the artery compressed, lay aside the scissors and pick up one of the canulæ.

(26) Holding the canula between the thumb and finger of the right hand insert the nozzle into the opening made in the artery. With a little practice this is easily done, and if a nozzle of a suitable size and shape is used the artery will contract round it so firmly that it will be difficult to remove it without damaging the vessel, and it is therefore quite unnecessary to tie the canula.

(27) The assistant who is giving the anæsthetic now takes by its lower third, one of the centrifuge tubes from the glass jar, carefully removes the cork with a pair of sterilised forceps, lays it on the sterilised towel, and holds the tube ready to receive the blood flowing through the canula when instructed to do so by the operator.

(28) By lifting the thumb slightly, release the compression on the vessel, and the blood should at once flow into the canula and begin to drip out through the end. Allow the first few drops to fall.

(29) Tell the assistant to place the centrifuge tube under the end of the canula between the falling drops, so that no blood touches the sides, but the blood falls into the opening of the tube straight to the bottom.

(30) The tube is held in this position until it is about three parts full of blood. The blood usually comes from the canula

in a succession of quick drops, not in a stream, and it takes about a minute or a minute and a half to collect sufficient blood in a tube holding 10 c.c.

(31) While the blood is being collected the anæsthetic must be carefully watched and no movement of the bird allowed.

(32) As soon as sufficient blood is collected in the centrifuge tube replace the cork quickly and place the tube in the ice. Great care must be taken that the cork does not touch anything non-sterile between the time of its being taken from the mouth of the tube to being returned to it again.

(33) In a similar way collect blood in the other centrifuge tubes until a sufficient supply has been collected, or until the greater part of the blood has been withdrawn from the bird. The assistant now deepens the anæsthesia so that the bird is killed. (It is possible to remove the bulldog forceps, tie the artery, and sew up the wound and put a collodion dressing upon it, and then return the bird to the pen, and thus avoid killing it.)

(34) If during the operation the blood runs badly it can usually be made to flow freely again by sponging the vessel with a swab dipped in the hot saline, if in spite of this, the blood for any reason ceases to flow, a pair of bulldog forceps should be placed on the artery below the canula and the other artery then be used for the supply.

(35) After the operation is finished the assistant should at once remove the bird, operating board, towels and instruments from the culture room.

(36) The towels should be washed and dried ready for sterilising when required again, and all instruments should be carefully washed in hot water and thoroughly dried.

(37) The paraffined pipettes are thrown away. Any spare glass canulæ should be returned to the tube containing olive oil.

Blood may be obtained from an artery of any animal if of suitable size, by the above method.

SECTION XXVII

To Separate Plasma from Red Blood Corpuscles

(1) Place in the buckets of the centrifuge the centrifuge tubes into which the blood has been received (Section XXVI.) and pack crushed ice around them, being careful that the ice does not touch the mouth of the tube or cork.

(2) Balance the buckets carefully against each other and place them in the centrifuge.

(3) Be sure that the buckets swing freely, and that the corks do not catch against the bucket holder.

(4) Centrifuge at a moderately high speed until the plasma is separated from the blood corpuscles. About 5 minutes is usually long enough.

(5) Remove the buckets from the centrifuge, lift out the centrifuge tube, and note if the red blood corpuscles have completely separated, leaving a deep layer of clear yellowish plasma. If the plasma is not clear it is either because the centrifuge has not been running smoothly, or because it has not been centrifuged long enough.

(6) Place the centrifuge tubes in the ice in the glass jar.

(7) Take one of the sterile corked and paraffined cold storage tubes from the ice and also one of the centrifuge tubes containing the separated plasma. Hold the two tubes between the thumb and index finger of the left hand so that they do not touch each other.

(8) Carefully remove the corks with sterile forceps and lay them on the sterilised towel.

(9) Take in the right hand one of the sterile paraffined pipettes already fitted with a teat, draw off some of the clear plasma, and transfer it to the empty paraffined tube. Repeat this until the greater part of the plasma has been decanted. Do not take the last few drops of plasma, as it is important that no red blood corpuscles should be drawn up. Be careful not to touch the sides of the tube with the pipette when removing plasma or when transferring it to the other tube.

(10) When the plasma has been decanted place the pipette on the sterile towel, and lay aside the centrifuge tube from which it has been decanted.

(11) Take up another cold centrifuge tube containing separated plasma and place it in the left hand, as before, together with the tube into which the plasma has been decanted; remove the cork with sterile forceps.

(12) Take up another paraffined pipette and transfer the plasma from the centrifuge tube to the tube containing the plasma. When sufficient plasma has been decanted lay aside the pipette on the sterile towel, and with the sterile forceps replace the cork in the tube containing the decanted plasma; replace this in the crushed ice and lay aside the tube containing the blood corpuscles. The amount of plasma placed in the paraffined cold storage tube depends partly on the amount drawn from the bird and partly on the size of the tube, but it is well to have two paraffined cold storage tubes and to halve the plasma between these, so that in the event of an accident happening to one, plasma is still available in the other.

(13) When sufficient plasma has been collected in the first cold storage tube replace the sterile cork with sterile forceps and return it to the ice in the jar.

(14) In the same way decant the remaining plasma into the second cold storage tube and then place it in the ice alongside the first.

(15) Take one of the Thermos flasks and half fill it with finely crushed ice.

(16) Take one of the sterile tubes fitted with a cork through which a string handle has been threaded, remove the cork, and with a pair of sterile forceps, place one of the tubes containing the decanted plasma into the tube, and cork tightly.

(17) With a pair of forceps, by means of the string handle, carefully lower the tube into the ice in the Thermos flask. With a little pressure and adjustment the tube will sink into the crushed ice.

(18) Add sufficient crushed ice to the Thermos flask to surround the tube to within one inch of the cork.

(19) Cork and cap the Thermos flask and stand aside.

(20) In the same way place the second tube of plasma in another Thermos flask.

SECTION XXVIII

To Transfer Plasma to a Capillary Pipette

(1) Open one of the Thermos flasks, and with a pair of forceps remove the container of the plasma tube from the flask by means of the string handle.

(2) Remove the cork of the container, and with a pair of sterile forceps carefully lift out the tube containing the plasma.

(3) With a pair of sterile forceps remove the cork from the plasma tube, and place it on a sterile surface.

(4) Take a sterile paraffined pipette (Section XXV.), and draw into it about $\frac{1}{2}$ c.c. of plasma without touching the side of the plasma tube.

(5) Place the pipette in a glass jar so that the end near the bulb rests on the roll plasticine (Section XI.).

(6) Recork the plasma tube, using sterile forceps, and place it in its container.

(7) Recork the container and replace it in ice in the Thermos flask.

(8) Cork and cap the Thermos flask.

SECTION XXIX

To Obtain Plasma from a Vein of a Bird

Where only small quantities of plasma are required this is a convenient method. The vein of the wing of a fowl is the best for a beginner to use.

The following apparatus is required: Operating board, length of bandage for tying fowl, ether, one pair sharp, stout

scissors, one pair fine scissors, two pairs dissecting forceps, one pair Dieffenbach's artery forceps, two pairs Spencer-Wells forceps (Fig. 26), liquid paraffin, a Record syringe fitted with a sharp needle of moderate size bore placed in a glass test-tube, the end of which has been drawn into a pipette slightly larger than the needle of the syringe, and the open end plugged with cotton wool, capillary pipettes and containers, teats, small centrifuge tubes with corks, small cold storage tube for containing plasma, in container with string loop through cork glass jars for powdered ice, a sterile simple saline solution, swabs, three sterile towels, a large roller bandage to support the wing, powdered ice, salt, and a can of paraffin wax.

(1) Sterilise the towels, corks, swabs, liquid paraffin, and simple saline solution in the autoclave, as already described in Section V.

(2) Sterilise the knives, scissors, forceps, syringe and needle in the hot water steriliser (Section II., 4).

(3) Sterilise the centrifuge tubes, capillary pipettes, and the glass containers for the syringe in the hot air steriliser.

(4) Paraffin the centrifuge tubes and the tube for containing plasma, as described in Section XXV., fit them with sterile corks, and place in ice. (Fig. 28.)

(5) Paraffin the pipettes, fit them with teats, lay them in their sterile containers and place them on ice.

(6) Remove the syringe from the hot water steriliser and fit together with sterile forceps; dry thoroughly first with absolute alcohol and then with ether; drawing a little sterile liquid paraffin, replace the syringe in the sterile container and embed in a mixture of powdered ice and salt, as the syringe must be ice cold when collecting the blood.

(7) Place the fowl, which should not have been fed for 24 hours, on the operating board, as in Section XXVI., being careful not to obstruct the flow of blood through the vessels of the wing from which the blood is to be obtained.

(8) Anæsthetise lightly with ether. Spread out the wing selected upon the roller bandage, which will act as a support, and carefully cut away the feathers from the neighbourhood

of the vessels running parallel with the humerus; do not pluck the feathers.

(9) With warm simple saline, sponge thoroughly the exposed area of the wing and the feathers around.

(10) With a pair of sterile forceps lift the skin over the vessels, and cut through this for about one inch and a half along the lines of the vessels.

(11) Retaining one edge of the incision in the forceps, clip the edge of the skin with a pair of Spencer-Wells forceps, and reflect it to one side. In a similar way with the other Spencer-Wells forceps reflect the skin on the other side. The vessels will now be exposed. With a blunt probe dissect out the vein and clip the distal end with a pair of bulldog forceps.

(12) Take the ice-cold syringe into which a little liquid paraffin has been already drawn, and plunge the needle into the vein parallel with its course and in the direction of the bulldog forceps. The assistant should at once release the pressure of the forceps and the blood will flow readily into the syringe on the plunger being gently withdrawn. When the syringe is full the assistant should at once release the bulldog forceps, and thus clamp the vessel.

(13) Withdraw the syringe and carefully discharge the blood into the paraffined centrifuge tubes in the ice. Do not force the blood from the syringe, but let if flow out in a gentle stream.

(14) Using sterile forceps. cork the centrifuge tubes, centrifuge, collect, and store the plasma as already described in Section XXVII. and XXVIII.

(15) The assistant should now remove the bulldog forceps from the vessel, and if any flow of blood takes place at the seat of puncture it is readily arrested by pressure. Bring the edges of the skin together with collodion and cotton wool, and turn the fowl into the pen.

Blood may be obtained from a large vein of any animal by this method.

SECTION XXX

To Obtain Blood from the Heart

If blood for plasma is required from rats, mice, guinea pigs, etc., it is most readily collected from the ventricles of the heart.

Apparatus required: Operating board, hammer and four 2-inch wire nails, a bell jar large enough to cover over the animal, wide-mouthed glass bottle with a little cotton wool that will fit easily over the head of the animal. Ether, barium sulphate paste, made with glycerine and water for removing the hair from the thorax, or, as an alternative, a sharp razor and soft soap, two sharp scalpels in test-tubes, one pair of sharp, stout scissors, four Spencer-Wells (Fig. 26a) forceps, three pairs of dissecting forceps, record syringe (the size of which will depend on the amount of blood likely to be obtained from the heart) with sharp, medium-sized needle, sterile liquid paraffin, sterile simple saline solution, sterile swabs, sterile towel, small centrifuge tubes coated with paraffin and fitted with corks, paraffined pipettes in containers. Crushed ice.

(1) Prepare, sterilise and set out the apparatus for the operation in a similar manner to that described in Section XXIX.

(2) Keep the animal without food for 12 hours, but allow plenty of water.

(3) Place the animal under the bell jar, pour some ether on a little cotton wool and insert this under the jar with the animal. In a minute or two the animal will become unconscious, and should at once be removed to the operating board and nailed out by its four paws, spread-eagle fashion, keeping it under the anæsthetic by means of ether on cotton wool in the wide-mouthed bottle.

(4) Remove the hair widely over the thorax and abdomen either with Barium paste or by shaving. Sponge the exposed surface freely with warm sterilised saline.

(5) With a sharp scalpel make a clean incision from the

neck to the pelvis, being careful not to cut through the muscles.

(6) With a pair of Spencer-Wells' forceps seize one edge of the incised skin, and with the aid of a fresh sterile knife reflect the skin from the thoracic abdominal muscles. With another pair of Spencer-Wells' forceps keep it reflected by allowing the two pairs to lie on the operating board beside the animal.

(7) In the same way reflect the skin on the other side of the incision.

(8) Take a pair of sterile scissors and dissecting forceps and cut the base of the thorax at the junction of the diaphragm with the sternum. Cut through the ribs on each side of the sternum, being careful not to wound the lungs or large vessels.

(9) With the aid of the forceps and a fresh sterile scalple reflect the sternum towards the neck and remove the reflected triangular portion by cutting through the apex with the scissors, again being careful not to wound any large vessel.

(10) The heart will now be exposed lying in the unopened pericardium. With a pair of sterile dissecting forceps and the fine scissors carefully cut into the pericardium and remove the anterior layer.

(11) With a fresh pair of sterile forceps seize the base of the heart gently and lift it up out of the pericardial sac.

(12) With a sterile swab moistened in saline, sponge any blood from the surface of the heart. Take the ice-cold syringe to which the needle has already been fitted and which already contains a little sterile liquid paraffin and plunge it into the ventricle, being careful not to penetrate the posterior wall.

(13) Quickly allow as much blood as possible to flow into the syringe, and when this is obtained at once discharge it without pressure into the ice-cold centrifuge tube. Replace the cork and centrifuge in ice immediately (Section XXVII.).

(14) With an ice-cold pipette, pipette off the upper two-thirds of the separate plasma and place in the ice-cold container (Section XXVIII.).

With small animals the plasma is generally used at

once, and it is therefore unnecessary to place it in the paraffined tube on ice, but if the plasma is to be stored it must be placed in ice in a Thermos flask as described in Section IX.

SECTION XXXI

To Obtain Frog Plasma

Apparatus required: Small cork mat, large pins, three small scalples, two pairs of fine-pointed scissors, three pairs of fine dissecting forceps, one pair of curved forceps, one pair sponge-holding forceps, four Spencer-Wells' (Fig. 26) forceps, six small capillary pipettes prepared from glass tubing with a lumen of $2\frac{1}{2}$ mm., rubber teats with small mouth, centrifuge tubes, glass flask containing sterile saline, powdered ice in glass jar, gas burner with small-pointed flame, small milk can three parts full of paraffin wax of melting point 56° C.

(1) Place the cork mat and pins on the bench in a good light.

(2) Sterilise the scissors and various forceps by boiling for 20 minutes (Section II. 4).

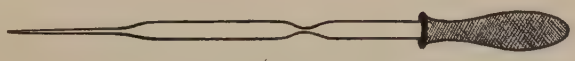


Fig 31

(3) Prepare capillary pipettes from glass tubing with outside diameter of about 4 mm. and a bore of about $2\frac{1}{2}$ mm. Cut this tubing into lengths of about 1 foot,heat the centre in the blow-pipe flame and draw out about 6 inches so as to make a capillary tube of fine bore.

(4) Cut the capillary through the centre, and thus make two capillary pipettes.

(5) Take one of these pipettes and heat again about $1\frac{1}{2}$ inches from the shoulder of the capillary tube and draw out for about $1\frac{1}{2}$ inches. Thus a pipette with a cylindrical bulb is formed (Fig. 31).

(6) With a fine-pointed gas flame (Section XX.) draw out the end of the pipette to a fine capillary, and with a

writing diamond cut the point so as to form a sharp pointed pipette. In a similar way prepare several pipettes.

(7) Place each pipette in a glass container (Section V., 2) and sterilise for half-an-hour at 130° C.

(8) Coat the pipettes with paraffin as directed in Section XXV.

(9) Fill the glass jar with finely powdered ice, and in this place upright two small centrifuge tubes also filled with powdered ice.

(10) Place a teat on each paraffined capillary pipette, and then replace in their sterile containers.

(11) Lay them on powdered ice, which is conveniently placed in a small enamelled pie-dish, taking great care that the powdered ice does not touch the opening of the container; the pipette in this way will be ice-cold when required.

(12) Sterilise the requisite instruments by boiling, and place them on the sterile towel on the bench in a convenient position.

(13) Take a large frog and wash it in a brisk stream of tap water.

(14) Etherise and pin out on the cork mat.

(15) Take a scalpel in the right hand and a pair of Spencer-Wells' forceps in the left.

(16) Clamp the skin in the middle of the abdomen, with the Spencer-Wells' forceps raise it gently, and make a clean incision through the skin the whole length of the ventral surface.

(17) Lay aside the knife and carefully pick up the skin on the other side of the incision with another pair of Spencer-Wells' forceps, and completely reflect the skin on both sides of the incision.

(18) Lay the forceps on the dissecting board in such a way that the skin will be held reflected from the exposed ventral surface.

(19) Care must be taken not to allow any moisture to run from the skin on to the exposed surface.

(20) With a fresh knife make an incision so as to expose fully, but not to cut into, the heart and large vessels.

(21) Lay aside the knife and reflect the abdomen walls with Spencer-Wells' forceps in the same way as with the skin.

(22) With a pair of sharp scissors and dissecting forceps open the pericardium.

(23) Lay aside the scissors, and with the pair of curved forceps seize the heart gently at the base and lift it from the pericardial cavity.

(24) Take one of the ice-cold sterile capillary pipettes express a small quantity of air from the teat and plunge the capillary end into the ventricle of the heart, being careful not to penetrate the posterior wall of the ventricle.

(25) Draw up as quickly as possible the blood from the ventricle until no more flows, or until the bulb of the pipette is two-thirds full.

(26) Remove the pipette, and by gently releasing the teat draw the blood which is in the capillary portion into the bulb, but do not allow air to enter the bulb.

(27) Maintain the pressure on the teat carefully so that the blood neither returns into the capillary, nor runs into the upper constricted portion of the pipette. Unless the pipette is held very steadily the blood will flow backwards and forwards into the capillary. This must be avoided.

(28) Allow the heart to fall back into the pericardial sac, lay aside the forceps, and, taking the capillary end of the pipette in the left hand, seal it off in the fine pointed gas flame about half-an-inch below the bulb, being very careful not to heat the blood in the bulb.

(29) The pressure on the teat may now be released and the constricted portion above the bulb is sealed off in a similar way, again taking great care not to heat the blood.

(30) The sealed off bulb is now rapidly plunged into the powdered ice in one of the centrifuge tubes.

(13) Place the centrifuge tubes in their buckets, balance exactly, and centrifuge at about 2000 revolutions per minute until the red corpuscles are thrown down and the clear plasma separated out. Five minutes is usually sufficient.

(32) Remove from the centrifuge and place the tubes in powdered ice until required.

SECTION XXXII

To Obtain Human Plasma

The blood may be taken from the median besilic vein.

(1) Apparatus required: A long, sharp, large-size bore record syringe needle in sterile olive oil in a test-tube, a length of roller bandage, a sterilised towel, a small bottle of ether, cotton wool, ice-cold paraffined centrifuge tubes with cork, paraffined pipettes fitted with teats, and in a glass container on ice two centrifuge tubes, sterile forceps and swabs.

(2) Place in the drum of the autoclave the towel, the corks for the centrifuge tubes, which should be arranged in a piece of gauze, and the hypodermic needle in the olive oil in the test-tube and autoclave at a temperature of 120° C. for 30 minutes.

(3) Sterilise the forceps by boiling for 20 minutes and then allow them to dry under the sterilised towel, which is placed folded in half on a table beside the donor of the blood.

(4) The centrifuge tubes and pipettes are paraffined as described in Section XXV., and with the corks and teats fitted, are placed on ice as described in Section XXIV. and placed ready at hand.

(5) When all is ready the donor of the blood is seated in a comfortable chair in a good light and the arm carefully cleaned over the median besilic vein with soap and water, dried with a sterile swab, and then washed with ether. Place a dry sterile swab over the vein; the donor can hold this by placing one finger upon it. The operator's hands are washed absolutely clean with soap and water for 5 minutes, or sterile rubber gloves may be used.

(6) Tie the strip of roller bandage round the donor's arm just above the biceps.

(7) The assistant should now hold the ice-cold centrifuge tube ready to receive the blood as it flows from the hypodermic needle.

(8) Remove the hypodermic needle from the oil in the test-tube with sterile forceps and gently shake out the oil from the needle.

(9) Hold the needle firmly with the thumb and finger at its lower third, remove the swab covering the vein, and insert the needle obliquely into a vein at the bend of the elbow, which by this time should be, at the bend of the elbow, distended with blood.

(10) The blood will now begin to flow. The first few drops should be allowed to fall on a piece of gauze.

(11) Now collect as much blood as is required in the cold centrifuge tubes, being careful not to allow anything to touch the end of the hypodermic needle.

(12) When the tubes are about two-thirds full, cork, and replace them in the ice. Allow the blood to run while changing one filled tube for another.

(13) As soon as enough blood has been obtained at once release the bandage and withdraw the needle, place the sterile swab over the puncture and bandage lightly.

(14) Place the tubes containing the blood in centrifuge buckets in ice, balance and centrifuge (Section XXVII.).

(15) Draw off the upper two-thirds of the plasma into the ice-cold paraffined pipettes and put up the required cultures at once.

Human plasma does not keep well, and should be obtained fresh on every time it is required.

Plasma may be obtained from the dog or other large animal by a technique similar to that used for the human subject.

SECTION XXXIII

To Cultivate Tissues in Plasma

The most suitable tissue for a beginner is the heart of an embryo chick of about 10 days' incubation.

(1) Set out the bench as described in Section XI.

(2) Remove the embryo from its shell (Section XIII.), and remove and cut up the tissues required for cultivation as described in Section XV.

(3) With a pair of sterile fine dissecting forceps place four coverslips on the right-hand side of the glass plate over the piece of black paper lying under it.

(4) Take from the glass jar the capillary pipette containing the plasma, and holding it perpendicularly, gently press the teat until a column of plasma fills the capillary portion and a drop appears at the end of the pipette.

(5) Allow the drop to fall gently on the centre of one of the coverslips.

(6) In the same way place a drop of plasma on the three remaining coverslips.

(7) With the blade of a sterile cataract knife transfer the fragment of the tissue to be cultivated to the centre of the plasma on the coverslip.

(8) With a circular movement of the point of the knife spread the plasma round the fragment so that it forms a circle about $\frac{1}{2}$ inch in diameter. The fragment of tissue should lie directly upon the coverslip.

(9) In the same way embed a fragment of tissue in the plasma on the other three coverslips.

(10) Replace the sterile Petri capsule over the four coverslips and allow the plasma to clot.

The time required for the formation of a firm clot varies with different samples of plasma, also with the species of animal from which the plasma was obtained.

(11) When the plasma has clotted firmly, invert the coverslip over the hollow of one of the ground slides, seal with melted paraffin, and incubate as directed in Section XVI., 6 to 15.

For some experiments it may be desirable to implant the fragment of tissue upon the surface of the mixture of plasma after clotting has taken place. This can be readily done by placing the fragment on the surface of the clotted plasma with the point of a sterile cataract knife, the culture being then inverted over the hollow of the slide, sealed and incubated as above.

SECTION XXXIV

To Cultivate Tissues in Plasma and Embryo Extract

The most suitable tissue for a beginner is the heart of an embryo chick of about 10 days' incubation.

(1) Set out the bench as directed in Section XI., putting out an extra capillary pipette with teat.

(2) Prepare the tissues for cultivation (Section XV.), and make the embryo extract as directed in Section XVII.

(3) Transfer a little plasma to a capillary pipette as directed in Section XXVIII.

(4) Take a fresh capillary pipette from the glass jar and carefully removing the cork from the tube containing the embryo extract, draw up about $\frac{1}{2}$ c.c. of the supernatant fluid into the pipette.

(5) Replace the pipette in the sterile glass jar.

(6) With a pair of sharp pointed sterile forceps place four coverslips on the right-hand side of the glass plate over the piece of black paper lying under it.

(7) Take the capillary pipette containing the plasma, and, holding it perpendicularly, press the teat until a column of plasma fills the capillary portion and a drop appears at the end of the pipette.

(8) Allow the drop to fall gently on to the centre of the coverslip.

(9) In the same way place a single drop of plasma on the other three coverslips.

(10) With the blade of a sterile cataract knife place a fragment of the tissue in the centre of the drop on each coverslip.

(11) Take the pipette containing the embryo extract from the glass jar.

(12) As quickly as possible allow a drop of the embryo extract to fall gently on each of the four drops of plasma.

(13) Replace the pipette in the sterile glass jar.

(14) With the point of a sterile cataract knife mix the plasma and extract and spread the liquid as rapidly as possible around each fragment in such a way as to form a circle about $\frac{1}{2}$ inch in diameter. The fragment should lie upon the coverslip. It is necessary to work quickly, otherwise clotting may take place before the plasma and extract have been properly mixed and spread out around the fragment.

(15) Place a sterile Petri capsule over the four coverslips

(16) When a firm clot has been formed invert each coverslip over the hollow of a ground slide, seal with melted paraffin, and incubate as directed in Section XVI., 6, 15.

For some experiments it may be desirable to implant the fragment of tissue upon the surface of the mixture of plasma and embryo extract after clotting has taken place. This can be readily done by placing the fragment on the surface of the mixture of plasma and embryo extract with the point of a sterile cataract knife, the culture being then inverted over the hollow of the slide and sealed and incubated as before.

SECTION XXXV

To Make Sub-cultures of Tissues Growing in Plasma or Plasma and Embryo Extract

The plasma used in sub-culture is usually that of the animal from which the tissues were originally obtained, or freshly collected plasma of an animal of the same species.

(1) Set out the bench as for putting up the original cultures.

(2) Place a little saline in the hollow of a ground slide and cover with a Petri dish.

(3) Place a sterile coverslip under a Petri dish.

(4) Take one of the cultures, examine under the microscope, and if growth is present note the amount of growth and the arrangement of the cells. Also be sure that the culture is not infected.

(5) With a sharp scalpel remove the coverslip from the slide by running the knife round the edges of the coverslip under the paraffin; care and practice are required for this, otherwise the coverslip will be broken.

(6) Remove as far as possible any of the paraffin adhering to the coverslip, being careful not to allow any fragments or dust of the paraffin to fall on the plasma in which the culture is embedded and lay the coverslip on the small black glass square on the glass plate, culture-side upwards.

(7) Hold the coverslip by the edges between the thumb and finger of the left hand and cut through the outer cells at the periphery of growth with a very sharp, sterile, cataract knife. To do this neatly a clean cut should be made on one side of the fragment, the coverslip turned a quarter round and a similar cut made at right angles to the first in such a way as to cut through a few cells of the outer part of the growth.

(8) Again turn the coverslip a quarter round, and make a similar cut through the edge of the culture at right angles to the second.

(9) Once more turn the coverslip and make a cut through the edge of the culture, which will cross the lines of the first and third incision. In this way the culture has been cut into a small square (the size of which will vary with the size of the culture), and a few cells will be left outside the line of each incision.

(10) With the blade of the cataract knife pick up carefully the small square of plasma and culture and place it in the saline on the hollow-ground slide. It is important not to take over any of the plasma laying outside the growth with the sub-culture.

(11) Gently wash the fragment in the saline by moving it backwards and forwards with the point of the knife.

(12) Replace the cataract knife in the jar, and place a drop of the plasma on the sterile coverslip already put out (Section XXXIV.).

(13) Transfer the culture to the plasma, being careful to allow it to float out flat. If tissue extract is being used for culture add one drop of the extract (Section XXXIV.).

(14) Quickly mix and spread the medium in a circle round the fragment, so as to leave the tissue sub-cultured spread out on the coverslip in the centre of the film (Section XXXIV.).

(15) The coverslip is then covered with a Petri dish until the clotting takes place.

(16) When clotting is firm, invert the culture over a hollow-ground slide and seal with paraffin.

(17) Label carefully and place in the incubator at the desired temperature.

SECTION XXXVI

The Cultivation of Frog's Tissue in Plasma

To obtain tissue for cultivation it is important to remember that only tissues which are sterile can be used, therefore with the frog the skin and intestinal track cannot be used; the other tissues and organs, however, are available.

The dissection of the tissues must be most carefully made and the skin widely reflected from the tissue selected. Great care must be taken not to wound the intestine, otherwise the infected contents will cause contamination.

(1) Set out the bench as described for the cultivation of tissues in saline solution (Section XI.).

(2) With a sharp cataract knife cut up the tissues selected and place them in a little saline solution on a hollow-ground slide.

(3) Remove one of the tubes containing the centrifuged blood plasma from the ice. With a sharp glass-cutting knife cut off the upper end of the tube and immediately re-embed this in the powdered ice.

(4) Place four sterile coverslips on the glass plate to your right, covering them with a Petri dish.

(5) Take the small fragments of tissue to be cultivated and wash them in fresh saline placed on a second hollow-ground slide.

(6) With a fine capillary pipette remove a little of the clear plasma from the tube, taking great care not to suck any blood into the pipette. Quickly place a small drop of plasma on each of the four coverslips and replace the pipette in the sterile jar.

(7) With the point of a cataract knife place a fragment of the tissue on each drop of plasma on each of the coverslips.

(8) Quickly spread out the drop of plasma round the fragment in such a way that the fragment lies on the glass surface in the centre of the film. Cover with a Petri dish.

(9) When the plasma has clotted, invert each coverslip over a hollow-ground slide, and seal with paraffin wax (Section XVI., 9 to 15). Label the slides and keep at room temperature.

If more cultures are desired put them up in a similar way. The tissues naturally grow more slowly than those of warm-blooded animals, and it may be a week to 14 days or longer before growth of the tissue cells commences. If red blood cells or lycocytes are present in the implant, these begin to wander out into the plasma within an hour or so after the tissues are put up, and must not be mistaken for growing cells.

It is not desirable to attempt to store the plasma left over, as it does not keep well. Fresh plasma should be obtained whenever it is necessary to put up fresh tissues, or to make sub-cultures of those already put up.

SECTION XXXVII

Intra Vitam Staining of Cultures

Cultures grown in any form of medium may be stained by intra vitam methods, but the best results will be obtained

with those grown in saline alone or in saline and embryo extract. For demonstration of cell granules other than the mitochondria neutral red is recommended, but other intra vitam stains may be used. A dilution of 1 in 80,000 will stain the granules, but for simple demonstration of these it is not necessary to make up a measured dilution. A solution of the stain in saline of such strength that when placed in a test-tube the colour is clearly seen while the light is readily transmitted, will give excellent results.

To stain the cultures.

(1) Remove the culture from the incubator, and while the paraffin is warm raise the coverslip from the hollow-ground slide by running the blade of a scalple under the surrounding wax.

(2) With a capillary pipette insert sufficient of the selected stain to fill the hollow. Replace the coverslip and the culture will thus be immersed in the stain.

(3) Place under the microscope on a warm stage, and within a few minutes the granules will begin to take up the stain.

(4) The staining of the granules and cell gradually becomes more intense, but within an hour or so the stain generally becomes diffuse.

SECTION XXXVIII

Intra Vitam Staining of Mitochondria

For the demonstration of mitochondria janus green will give perfect results, but only if the stain is of the correct formula. E. V. Cowdrey[1] states that this is diethylsafraninazodimethylanilin chloride janus green B (Farbwerke Hoechst Co.). He also states that janus black is a specific stain for mitochondria in living blood cells, but that it is not a pure dye and owes its specificity to the janus green it contains.

[1] *The American Journal of Anatomy*, Vol. XIX., No. 3, May, 1916.

A dilution of 1 in 40,000 is often used, but it is unnecessary to make up a measured solution for simple demonstration of the mitochondria. A solution of the stain in saline is a test-tube similar to that described for neutral red will give an excellent preparation.

The cultures should be stained as described in Section XXXVII., and watched under a 1-12 oil objective on a warm stage. The mitochondria gradually take up the stain, but after an hour or so the staining becomes diffuse. The solution of the stain may be mixed with a similar solution of neutral red, and in this way both the cell granules and mitochondria can be stained simultaneously.

SECTION XXXIX

Fixation of Cultures in Vitro for Staining

Cultures in vitro are usually fixed and stained on the coverslip on which they are growing, although cultures in plasma may be sectioned and stained after fixation.

Cultures grown in saline solution or in saline and tissue extract may be fixed by any of the usual fixatives, but care must be taken not to over-fix the cells, therefore the time usually allowed should be shortened.

Cultures grown in plasma or plasma and tissue extract are more difficult to fix, as the plasma is rendered opaque by many of the fixatives in common use; therefore, if such sections are prepared it may be difficult to clear the specimen after staining.

It is also important if the cultures are fixed in watery solutions and afterwards brought into alcohol, either before or after staining, to bring them very slowly through gradually increasing strengths of alcohol, otherwise shrinkage and distortion of the cells may take place. A good method is to place the coverslips in a Petri dish, culture side uppermost, in a little of the fixative or in water if they have been washed after fixation, then from a suitable bottle allow 70 per cent. alcohol to fall drop by drop at the rate of about 1 drop per 30 seconds until the fixative or water is practically replaced

by the 70 per cent. alcohol. Then pour off most of this from the Petri dish and allow absolute alcohol to fall drop by drop at a similar rate until the strength required is almost obtained; then pour off all the alcohol and replace it by absolute alcohol. The Petri dish should be gently tilted backwards and forwards every few minutes to mix the contents. This method is slow, and requires patience, but the results are worth the trouble.

SECTION XL

To make an Aqueous Solution of Hæmatoxylin

One per cent. solution of hæmatoxylin in freshly prepared distilled water from a glass retort.

Use a clean-stoppered bottle and place the mixture in a south window for 2 weeks, shaking occasionally. The stain keeps for about 2 months; after this time is apt to stain deficiently. It should therefore be prepared in small quantities at regular intervals.

SECTION XLI

To make Iron Alum Solution

Dissolve $2\frac{1}{2}$ grams of iron alum (ammonia-ferric sulphate) in 100 c.c. of distilled water. The iron alum crystals must be of a clear violet colour and kept in a well-stoppered bottle. Do not use dull or yellow crystals. Allow them to dissolve at room temperature.

SECTION XLII

Scharlack R Solution

Equal parts of acetone and 70 per cent. alcohol saturated with Scharlack R, filter before use.

SECTION XLIII

Acetic Alcohol

0·5 per cent. glacial acetic acid in 70 per cent. alcohol.

SECTION XLIV

Fixation in Acetic Alcohol and Staining by Iron Hæmatoxylin

Apparatus required: Acetic alcohol (Section XLIII.), solution of iron alum (Section XLI.), 0·5 per cent. aqueous solution of hæmatoxylin (Section XL.), glass capsule, Petri dishes, glass slides, forceps, small scalpel, labels, saline solution.

(1) Remove the culture from the incubator, and while warm remove the coverslip from the hollow-ground slide by running the blade of a scalpel under the wax.

(2) If the culture has been grown in plasma medium, place the coverslip for 5 minutes into saline solution kept at a temperature of 37° C.

(3) Place the culture in a watch glass containing acetic alcohol for 5 minutes.

(4) Wash in running distilled water for 5 minutes.

(5) Place in 2½ per cent. iron alum solution for 30 minutes at room temperature.

(6) Wash in running distilled water for 5 minutes.

(7) Take the hollow-ground slide on which the culture has been grown, wipe it clean and free from wax, and with a clean glass pipette fill the hollow with 0·5 per cent. hæmatoxylin solution.

(8) Remove the coverslip from the distilled water and invert it, culture-side downwards, upon the hæmatoxylin, in the hollow of the ground slide.

(9) Place in the incubator for 5 minutes.

(10) Remove the slide from the incubator and the coverslip from the slide and wash the coverslip in distilled water for 2 or 3 minutes.

(11) Differentiate in 2½ per cent. iron alum. As the differentiation progresses the specimen must be carefully watched under the microscope. This is best done by removing it from the iron alum, washing in distilled water and

placing it, culture-side upwards, on the glass slide. Differentiation must be continued until the cells are clear and the nucleoli or the chromosomes of the cells in mitosis if present, stand out clearly.

(12) When the culture is sufficiently differentiated wash in running distilled water for 5 minutes.

(13) Mount directly into Farrant's mounting medium and label.

If preferred, the specimen may be passed through the alcohol and xylol and mounted in Canada balsam.

SECTION XLV

To Stain Cultures for Fat

(1) Proceed as described in Section XLV., but after differentiation and washing in distilled water place the specimen in a solution of Scharlack R (Section XLIII.) for 5 minutes, or until examination under the microscope shows the fat is well stained.

(2) Wash in distilled water.

(3) Mount in Farrant's mounting medium.

SECTION XLVI

To Prepare Bouin's Picro Formol Solution

Picric acid saturated solution in distilled water 75 parts, formol 25 parts, glacial acetic acid 5 parts.

SECTION XLVII

Fixation of Cultures by Bouin's Solution and Staining with Iron Hæmatoxylin

One per cent. solution of hæmatoxylin in distilled water, solution of iron alum, Bouin's picro-formol solution, absolute alcohol, xylol, Canada balsam, distilled water, saline solution, glass slides, fine forceps, watch glasses, glass capsules, Petri dish, labels, small scalpel.

(1) Remove the culture from the incubator, and while the paraffin is warm lift it from the hollow-ground slide by running the edge of the scalpel under the wax round the coverslip.

(2) Lift off the coverslip, remove any wax adhering to it with the scalpel. This requires care, as the coverslip is easily broken.

(3) If the culture has been made with plasma, wash the coverslip gently for 5 minutes in saline solution kept at a temperature of 37° C. This is best done by placing some Ringer solution in a Petri dish in the incubator about half-an-hour before beginning to stain.

(4) Place the culture in Bouin's solution in a watch glass. Cover with a Petri dish and allow to remain in fixative for 15 minutes.

(5) Remove the coverslip from the Bouin's solution (Section XLII.) into running, distilled water for 5 minutes.

(6) Take the hollow-ground slide on which the culture has been grown, wipe it clean, and free from paraffin wax, and with a clean glass pipette fill the hollow with a 0·5 per cent. hæmatoxylin solution (Section XL.).

(7) Place the coverslip into a 2½ per cent. solution of iron alum (Section XLI.) in a watch glass, allow it to remain in this for half-an-hour.

(9) Remove the coverslip from the iron alum solution and wash in running distilled water for 5 minutes.

(10) Remove the coverslip from the distilled water, shake off excess and invert it, culture-side downwards, upon the hæmatoxylin in the hollow of the glass slide.

(11) Place the slide in the incubator for 5 minutes.

(12) Remove the slide from the incubator and the coverslip from the slide and wash the coverslip in running, distilled water for about a minute.

(13) Differentiate in 2½ per cent. iron alum. As the differentiation progresses the specimen must be carefully watched under the microscope. This is best done by removing it from the iron alum washing in distilled water and placing it, culture-side upwards, on a glass slide. Differential

tion must be continued until the cells are clear and the nucleoli and the chromosomes of the cells in mitosis, if present, stand out clearly.

(14) When the culture is sufficiently differentiated wash in running, distilled water for 5 minutes.

(15) Put out six glass capsules and number them 1 to 6.

(16) Into No. 1 put 70 per cent. alcohol.

(17) Into Nos. 2–3 and 4 put absolute alcohol.

(18) Into Nos. 5 and 6 put xylol.

(19) Cover each capsule over with a Petri dish.

(20) Remove the stained culture from the distilled water and place in capsule No. 1 for 5 minutes.

(21) Transfer to capsules No. 2, 3, 4, 5 and 6, allowing the section to remain 5 minutes in each, keeping the capsules covered.

(22) Mount in Canada balsam and label.

Printed by
W. Heffer and Sons Ltd.,
Cambridge, England.

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